# Investigation of the Role of Disproportionating Enzyme in Starch Metabolism by Isolation and Characterisation of a Mutant of *Arabidopsis thaliana* (L.)

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## Declaration

I declare that this thesis was composed by myself, and that the work contained within it is my own, unless otherwise stated.

Joanna Harriet Critchley
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## Abbreviations and Glossary

| | |
|---|---|
| (v/v) | volume:volume ratio |
| (v/w) | volume:weight ratio |
| °C | degree Celsius |
| A | absorbance |
| ADP | adenosine 5'diphosphate |
| ADP-Glc | ADP-glucose |
| AGPase | ADP-glucose pyrophosphorylase |
| APS | ammonium persulphate |
| ATP | adenosine 5'triphosphate |
| BE | branching enzyme |
| bp | base pair |
| BPB | bromophenol blue |
| BSA | bovine serum albumen |
| cDNA | complementary DNA |
| Ci | Curie |
| CTAB | hexadecyltrimethylammonium bromide |
| Da | Dalton |
| dCTP | 2'deoxycytidine 5'triphoshate |
| DEPC | diethyl pyrocarbonate |
| D-enzyme | disproportionating enzyme |
| DNA | deoxyribonucleic acid |
| dNTPs | deoxynucleosidetriphoshates |
| DP | degrees of polymerisation |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetra-acetic acid (disodium salt) |
| EST | expressed sequence tagged DNA |
| FLB | Feldmann left border |
| FRB | Feldmann right border |
| GBSS | granule-bound starch synthase |
| G-1-P | glucose-1-phosphate |
| HEPES | *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethane sulphonic acid) |
| IPTG | 1-thio-β-D-galactopyranoside |
| Mr | relative molecular mass |
| mRNA | messenger RNA |
| MES | 2-(*N*-morpholino)ethanesulphonic acid |
| MOPS | 3-(*N*-morpholino)propanesulphonic acid |
| MOS | malto-oligosaccharides |
| NADPDH | nicotinamideadeninedinucleotidephosphate dehydrogenase |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| PVPP | polyvinylpolypyrrolidone |
| RNA | ribonucleic acid |
| RNase | ribonuclease |

| | |
|---|---|
| polyA$^+$ | polyadenylated |
| rpm | revolutions per minute |
| SDS | sodium dodecyl sulphate |
| SSC | sodium chloride/sodium citrate |
| SS | starch synthase |
| SSS | soluble starch synthase |
| TBS | Tris buffered saline |
| TBE | Tris/Borate/EDTA |
| T-DNA | transfer DNA |
| $T_i$ | tumour inducing |
| TE | Tris/EDTA |
| TEMED | N,N,N',N'-tetramethylethyldiamine |
| TNS | trinitrosalicylic acid |
| Tris | tris-(hydroxymethyl)-methylamine |
| TWEEN-20 | polyoxyethylenesorbitanmonolaureate |
| Ws | *Arabidopsis* ecotype Wasserilewskija |
| wt | wild-type |
| X-Gal | 5-bromo-6-indolile-β-D-galactoside |

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# Chapter 1

# Introduction

## 1.1 Starch and its structure

### 1.1.1 Starch and energy storage

A supply of energy is required by all living things to carry out their life processes. Since the supply and the demand for energy varies over time, a means of storing the energy is fundamental to almost all biological systems. Starch and glycogen are large carbohydrate molecules used as a means of storing the energy of sugars safely by cells for times when it is needed. Chemically, they are simple polymers consisting solely of glucose residues linked in α (1-4) and branched in α (1-6) positions. Cells from all the biological kingdoms contain either or both of these polymers, from the most primitive bacteria and algae to the cells of the human body.

Starch is the carbohydrate reserve used by most higher plants - thus it is one of the world's most abundant biological polymers and the main source of energy in the human and animal diet. Starch differs from glycogen in the complexity with which it is packed. The branching of glycogen occurs in a seemingly unregulated way until steric hindrance prevents the molecule from becoming any larger. Starch packs efficiently into complex granules ranging in size from 0.2 μm to >100 μm containing highly ordered crystalline and semi-crystalline zones. The similarity between glycogen and starch in terms of chemistry and metabolism means that information obtained in the study of quite diverse organisms may be of help in elucidating the formation and metabolism of starch granules and what factors determine the differences between starches.

Starch has two components: amylose which contains very few α (1-6)-linked branches and amylopectin which contains branches at regular intervals (Hizakuri, 1986 and 1996). Amylopectin is the major component and is considered the sole molecular fraction required to generate normal granules. The amount of branching and the ratio of amylose to amylopectin varies between species, organ and developmental stage and these factors greatly influence the properties of the starch. Starch has been classified according to its role. Reserve starch is intended for long-term storage and is packed into amyloplasts (Martin and Smith, 1995), specialised storage plastids found in organs of perennation and dispersal, where it can provide

energy for the next generation or the next season's growth. In these organs it is abundant and most research on starch has concentrated on reserve starch in crop plants. Transitory starch is formed inside the chloroplasts during photosynthesis and then degraded in the following dark period for respiration and growth.

### 1.1.2 The observed structure of starch granules

Van Leeuwenhoek observed starch granules from wheat (*Triticum aestivum*) using the first microscope in 1719, and noted the concentric rings. These rings are alternating crystalline and amorphous lamellae, which are believed to reflect the arrangement of amylopectin chains into clusters. Figure 1.1 shows the structure within a starch granule. Within clusters, adjacent chains form double helices, which pack together in ordered arrays of inter-digitating helices to give the crystalline lamellae. The amorphous lamellae are the zones containing the α (1-6) branch points, hence few double helices are formed (Hizakuri, 1986; Jenkins *et al.*, 1993). The repeat distance between the rings is 9nm and this has been explained by the lengths of chains occurring within the clusters. The chain length distribution is polymodal, with maxima at 12-16, ~40 and ~70 glucose residues (French, 1984; Hizakuri, 1986). Chains within the granule are radially arranged with their non-reducing ends towards the surface: chains of 12-16 glucose residues are organised into clusters; chains of 40 residues span two clusters and chains of 70 residues span three clusters. One amorphous and one semi-crystalline zone together comprise what is known as a growth ring (Buttrose, 1960, 1963). It is thought that this zonal periodicity is determined by intermittent periods of synthesis for example the day-night regime. However, factors affecting the nature and size of growth rings are unclear. Growth rings in the starch of wheat endosperm are apparently abolished if the plant is grown in a constant environment (Buttrose, 1962). Potato (*Solanum tuberosum*) mini-tubers grown under constant light seem to maintain their growth rings (Emma Pealing-unpublished).

It is still unclear how amylose is arranged within the ordered amylopectin matrix. Mutant plants unable to synthesis amylose have structurally normal granules. Amylose molecules form single helical structures and they may exist interspersed with amylopectin in the amorphous regions.

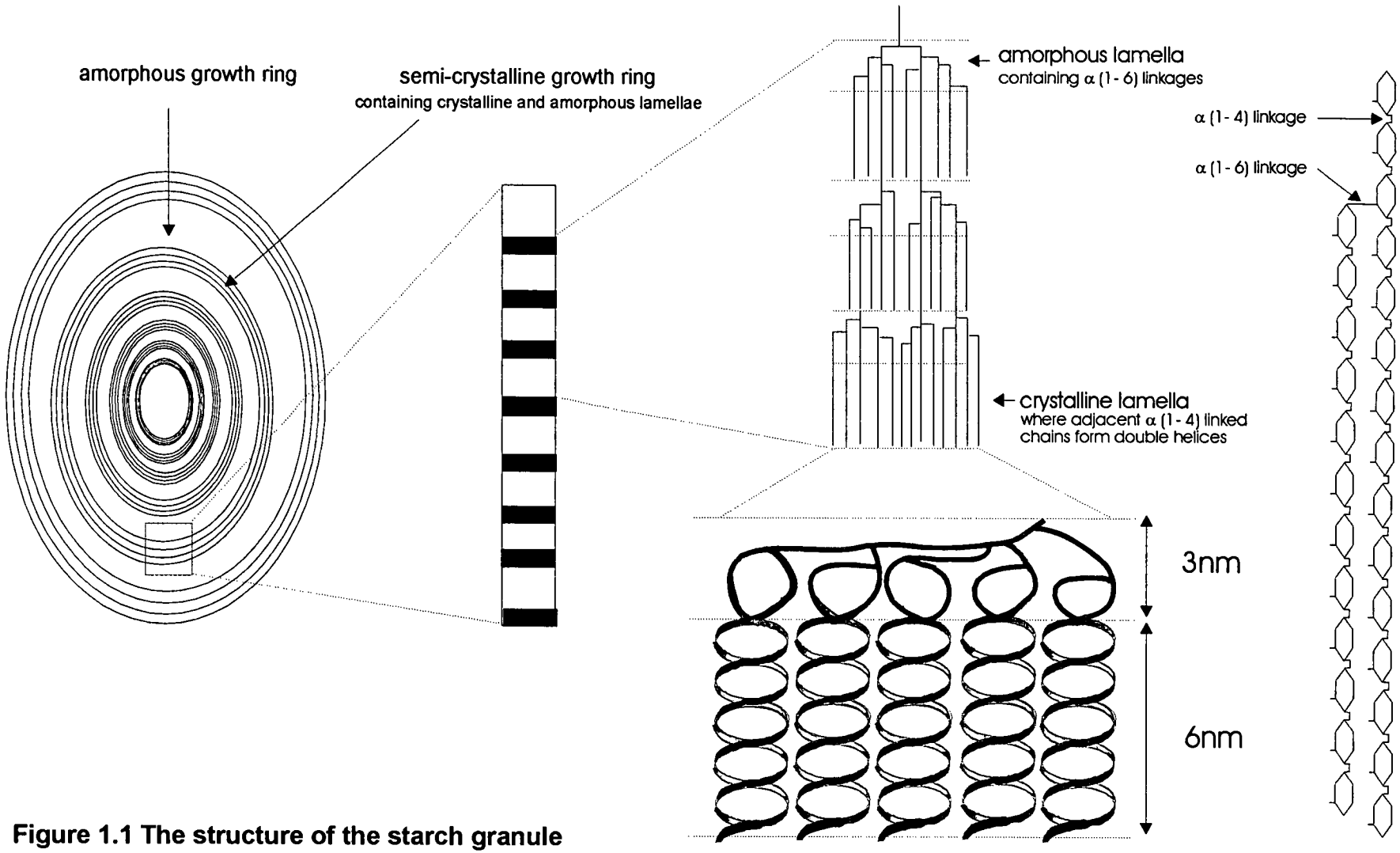



**Figure 1.1 The structure of the starch granule**

### 1.1.3 Transitory Starch

The pattern of starch accumulation in the leaves of many plants seems to have a strong diurnal rhythm where starch is synthesised during the day and degraded at night. *Arabidopsis thaliana* grown under a 12 h photoperiod degraded 85% of the accumulated starch in the subsequent dark period (Lin *et al.*, 1988). Since net synthesis and net degradation occur at discrete times during the course of a single day, the two processes can be studied within a short period without the complication of developmental and environmental changes

#### 1.1.3.1 Transitory Starch Granules

Transitory starch granules of chloroplasts are discoid in shape, flatter and considerably smaller than those found in amyloplasts. Though normally less than 5 μm across (Badenhuizen, 1969) starch granules from a single leaf were found to range in size between 0.2 and 5.5 μm in *Helianthus annuus* (Radwan and Stocking, 1957). The size of the granules may vary between tissue types within a leaf. In *Pellionia pulchra* leaves, the granules were found to be larger in the spongy mesophyll than the palisade layer (Weier, 1936).

Granules of transitory starch may have less crystalline order than storage starches (Radwan and Stocking, 1957). Starch granules isolated from *Spinacia oleracea* leaves have a crystalline core with an amorphous outer mantle consisting of lower molecular mass glucan polymers which is more readily degraded during the night (Steup *et al.*, 1983; Beck, 1985). Starch granules from *Nicotiana tabacum* leaves, however, contain amorphous and semi-crystalline zones similar to the growth rings of storage starches (Buttrose, 1963). The core and the material organised into growth rings may represent material that is not turned over regularly but instead accumulates gradually as the leaf ages. This material contains amylose and high molecular weight amylopectin and would be expected to be semi-crystalline. This slow-turnover leaf starch may have similarities to storage starch.

### 1.1.3.2 Chemical and compositional differences between transient and reserve starch

Chemical differences that probably contribute to differences in granule packing structure and metabolism between transitory and reserve starch are shown in Table 1.1.

**Table 1.1 A brief comparison of the transitory and reserve starch**

| | Transitory Starch | Reserve Starch |
|---|---|---|
| [Amylose:Amylopectin] | 16% or less Amylose | 20% or more Amylose |
| | (Matheson,1996; Radwan and Stocking, 1957; Taira *et al.*,1991; Hovencamp-Hermelink *et al.*,1988; Tomlinson *et al.*, 1997) | |
| Size of Amylose | Low molecular mass | High molecular mass |
| Structure of Amylose | More branched | Less branched |
| | (Matheson,1996; Radwan and Stocking, 1957) | |
| Polymodal Distribution of chain lengths of Amylopectin | in range 5-40<br>maxima at 12, 15 and 21 glucose residues<br>(Tomlinson *et al.*, 1997) | maxima at 12-16, 40 and 70 glucose residues<br>(French, 1984; Hizukuri, 1986) |

## 1.2 Synthesis of Transitory Starch

The total starch content of leaves from tobacco (*Nicotiana tabacum*) and cotton (*Gossypium* sp.) increases through development (Matheson, 1996; Chang, 1979). Amylose and amylopectin of higher molecular mass accumulate gradually as the leaf ages. During the day, as the starch content of the leaf rises, the molecular mass of amylopectin and the ratio of amylose to amylopectin falls (Matheson, 1996; Chang 1979). This, together with the chemical differences noted in Table 1.1 suggest that the diurnal changes in starch content in the leaf may be due mainly to the synthesis of a pool of low molecular mass amylopectin which can be rapidly turned over.

### 1.2.1 Pathway of starch synthesis in leaves

In the stroma of the chloroplast, fructose-6-phosphate derived from the reductive pentose phosphate pathway is converted to glucose-6-phosphate and then to glucose-1-phosphate via the enzymes phosphoglucoisomerase and phosphoglucomutase.

**ADP-glucose pyrophosphorylase** (EC 2.7.7.27, AGPase) catalyses the synthesis of ADP-glucose, the substrate for starch synthesis, from glucose-1-phosphate and ATP. The reaction is made irreversible by the subsequent hydrolysis of the other product of the reaction, pyrophosphate, by an alkaline inorganic phosphatase (Weiner *et al.*, 1987). **Starch synthase** (EC 2.4.1.21, SS) uses ADPglucose to add glucose to the non-reducing end of a growing linear chain. An α linkage is formed between the C1 on the glucose and the reactive C4 at the end of the chain and ADP is released. **Starch branching enzyme** (EC 2.4.1.18, SBE) forms the branch points in starch polymers by catalysing formation of an α (1-6) linkage between the C1 at the end of a linear glucan chain and a C6 of the main chain.

### 1.2.2 Starch structure and granule formation

#### 1.2.2.1 The role of starch synthetic enzymes in determining starch structure

It is accepted that the unique polymodal distribution of the chain lengths within the highly branched amylopectin component of the starch granule allows the chains to form double helices capable of packing into organised arrays giving rise to the semi-crystalline areas.

The specific chain-length profiles seen in starch polymers may be due in part to the existence of multiple isoforms of starch synthase and starch branching enzyme, although the existence of these isoforms is described better for storage organs than for leaves. SBE exists as two classes of isoforms (notated A and B) as distinguished by features of their amino-acid sequences (Burton *et al.*, 1995; Martin and Smith, 1995), which have distinct properties. In maize (*Zea mays*) endosperm, there are two different A isoforms and a B isoform. *In vitro* and when expressed in *E. coli*, the A isoform prefers more highly branched substrates and preferentially transfers shorter branches than the B isoform (Guan *et al.*, 1995; Guan and Preiss, 1993; Takeda *et al.*, 1993). The substrate preference and length of chains transferred are dictated by

differences in amino-acid sequence at the amino and carboxyl termini (Kuriki *et al.*, 1997). The two main forms of SBE purified from maize and rice (*Oryza sativa*) leaves have kinetic, antigenic and chromatographic properties similar to the A and B isoforms of endosperm (Dang and Boyer, 1988; Yamanouchi and Nakamura, 1992) although it is thought that the relative contributions of the two isoforms to the total activity may be different for leaf.

There has also been recent progress in understanding the nature and roles of starch synthase isoforms. It is accepted that one class of isoform that is tightly bound to the starch granule is specifically responsible for amylose synthesis. Mutant plants in which the activity of granule-bound starch synthase I (GBSS I) has been eliminated (for example, the *waxy* mutants of cereals, the *lam* mutant of *Pisum sativum*, the *amf* mutant of *Solanum tuberosum*, reviewed in Smith *et al.*, 1997) have starch consisting entirely of amylopectin. GBSS I in isolated starch granules of *P. sativum* elongates malto-oligosaccharides processively, adding more than one glucose molecule for each enzyme-glucan encounter (Denyer *et al.*, 1999). Amylose synthesis by GBSS I in potato tubers is wholly or largely confined to a central region of the granule, consistent with the idea that the space available in the matrix may be an important determinant of the amylose content of storage starches (Tatge *et al.*, 1999). Amylose content has been linked with changes in granule architecture also through experiments with enzyme-gold cytochemical markers (Atkin *et al.*, 1999). In the absence of amylose (waxy maize starch) a framework of closely packed concentric layers of amylopectin exists in the granules. Low amylose content (potato starch) results in alternating layers of densely packed amylopectin and amylose molecules. High amylose content (amylomaize starch) granules were shown to possess an amylopectin centre surrounded by an amylose periphery encapsulated by an amylopectin surface.

Multiple isoforms of soluble starch synthase that are responsible for synthesis of amylopectin have been identified. Differences in their amino-acid sequences define them as three distinct classes: SSI, SSII and SSIII. Information about the roles of the isoforms *in vivo* is resulting from studies of mutants lacking specific isoforms (Gao

*et al.*, 1998; Craig *et al.*, 1998). The *rug5* mutant of *P. sativum* has a mutation in the gene encoding the SSII isoform. Amylopectin produced by the mutant contains fewer intermediate length chains but more short chains, indicating that SSII may be responsible for synthesis of the intermediate length chains.

It is probable that the different enzymes and different isoforms work together in a synergistic way where the product of one is the substrate of another. Studies of transgenic potato tubers with reduced activities of either SSI and SSII or both together reveal that the effect of reduction of a particular isoform upon amylopectin depends upon the level of activity of the other isoform (Edwards *et al.*, 1999).

Another enzyme that may influence glucan structure is **debranching enzyme** (EC 3.2.1.41, DBE) which cleaves α (1-6) linkages. The sugary (*su1*) mutants of maize and rice which are deficient in DBE have reduced starch levels but contain a highly branched, soluble glucan called phytoglycogen (Doehlert *et al.*, 1993; Nakamura *et al.*, 1997). The gene at the *su1* locus in maize has been shown to encode a DBE of the isoamylase class (James *et al.*, 1995). DBE-deficient, phytoglycogen-accumulating mutants have also been found in *Chlamydomonas reinhardtii* (*sta7*) (Mouille *et al.*, 1996) and *Arabidopsis thaliana* (*dbe1*) (Zeeman *et al.*, 1998) and these groups have both proposed models to explain the role of debranching enzyme in amylopectin synthesis.

#### 1.2.2.2 New models of amylopectin synthesis

Recently several models have been proposed which attempt to fit the available information about the properties and putative roles of enzymes known to be involved with starch metabolism into a process which could result in the observed structure of the granule.

The 'pre-amylopectin trimming' model (Ball *et al.*, 1996) includes a direct role for debranching enzyme in determining the frequency and pattern of branches in amylopectin.

They propose a synthetic cycle as follows:

1. Starch synthase elongates short chains at the surface of the granule.

2. Branching enzyme branches these chains extensively to make 'pre-amylopectin'.
3. Debranching enzyme trims the over-proliferated branches leaving a few (close to the organised double helical zone which it cannot access) which will begin the next cluster.
4. These few will be elongated by starch synthase and the cycle continues.

This model provides an explanation for the cluster formation and the periodicity of the semi-crystalline layers, and also the presence of phytoglycogen. There is still no evidence that this is the process that occurs *in vivo*.

Zeeman and colleagues propose an alternative explanation for the accumulation of phytoglycogen. Since the *dbe1* mutant of *Arabidopsis thaliana* accumulates both phytoglycogen and normal amylopectin in its chloroplasts at the same time (Zeeman *et al.,* 1998), they propose that DBE is not directly involved in amylopectin synthesis. Instead they suggest that DBE, together with other starch metabolising enzymes, plays a scavenging role in the stroma degrading any soluble glucans produced by the action of starch synthase and SBE on malto-oligosaccharides. In the absence of DBE these soluble glucans may proliferate using the substrate and detain the enzymes that would normally be available at the granule surface for amylopectin synthesis. This results in phytoglycogen and in the production of less amylopectin.

#### 1.2.2.3 A role for starch phosphorylase in starch synthesis

*Solanum tuberosum* plastidic starch phosphorylase mRNA accumulates under conditions when starch biosynthesis is most active (in developing tubers and illuminated leaves) with a similar pattern of expression to starch synthetic enzymes, SBE (Koβmann *et al.*, 1991) and AGPase small subunit (Müller-Rober *et al.,* 1990) and also to D-enzyme (Takaha, 1996). Induction of a starch phosphorylase gene during starch synthesis has been reported in *Solanum tuberosum* (St-Pierre and Brisson, 1995). In *Vicia faba* the enzyme activities of both plastidic and cytosolic isoforms of starch phosphorylase correspond to the pattern of starch accumulation. Also, *in-situ* hybridisation reveals a correlation between gene expression of the plastidic isoform and starch granule formation in cotyledon development (Buchner *et al.*, 1996).

Glucan-phosphorylases catalyse the transfer to or the removal of a glucosyl moiety from an α-glucan – the protein is capable of acting as both a glucan-degrading activity and a glucan-synthesising enzyme. The ratio of glucose-1-phosphate to inorganic phosphate ($P_i$) determines whether the enzyme acts in the direction of synthesis or degradation. In *Vicia faba* amyloplasts, starch synthesis begins via AGPase which is subject to allosteric inhibition by $P_i$ with an inhibition constant, $K_i$ of AGPase for $P_i$ of 2 - 2.5 mM (Kleczkowski *et al.*, 1993; Weber *et al.*, 1995). Since the degradative pathway is optimal when the concentration of $P_i$ falls between 3 and 10 mM (Matheson and Richardson, 1978), the simultaneous synthesis of starch and its degradation by phosphorylase in the same compartment is unlikely.

Both starch phosphorylase and D-enzyme (discussed later) are generally thought to be involved in starch degradation. In *E. coli* both of these enzymes are encoded by genes on the same operon, *mal PQ*. There is growing evidence that both starch phosphorylase and D-enzyme, like debranching enzyme, may have a role in synthesis. Possible roles for starch phosphorylase in synthesis include production of glucan primers for starch synthases on the growing granule or degradation of soluble glucan in a role similar to that described for debranching enzyme in the Zeeman model.

#### 1.2.2.4 Regulation of starch synthesis

AGPase is allosterically regulated by 3-phosphoglycerate (3-PGA) and inorganic phosphate ($P_i$) and regulation of this enzyme may play a significant part of controlling carbon flux into starch synthesis (Preiss, 1991). The *adg1* and *adg2* mutants of *Arabidopsis thaliana* have only 2 and 5% (respectively) of wild-type AGPase activity and accumulated 2 and 40% of the starch found in wild-type leaves (Lin *et al.*, 1988a; Lin *et al.*, 1988b). Studies with isolated chloroplasts showed that a decrease in the supply of $P_i$ caused an increase in starch synthesis. It was proposed that this was mediated by decrease in the ATP/ADP ratio and hence a restriction of 3-PGA reduction. An increase in the 3-PGA/$P_i$ ratio in the chloroplast would lead to activation of AGPase and starch synthesis (Heldt *et al.*, 1977; Robinson and Walker, 1979). The concentrations of 3-PGA and $P_i$ are thought to vary with chloroplast

metabolism although this has proved difficult to measure *in vivo* (Gerhardt *et al.*, 1987).

The triose phosphate transporter present in the chloroplast inner envelope catalyses a 1:1 counter exchange of substrates and has similar affinities for 3-PGA and $P_i$ (Fliege *et al.*, 1978; Flügge and Heldt, 1991). It therefore plays a central role in determining the ratio 3-PGA: Pi between and within the cytosol and stroma of the chloroplast. When the rate of photosynthesis is low relative to the demand for sucrose, sucrose synthesis increases levels of $P_i$ in the cytosol, which is expected to result in export of triose phosphate from the chloroplast and a decrease in the 3-PGA to $P_i$ ratio. $P_i$ inhibits the activity of AGPase and carbon flux is preferentially diverted towards sucrose synthesis. When the rate of photosynthesis is high relative to the demand for sucrose, import of $P_{i\ from}$ the cytosol and export of triose phosphate from the chloroplast via the triose phosphate transporter is expected to be restricted, resulting in an increase in the ratio of 3-PGA to $P_i$ in the chloroplast. 3-PGA activates AGPase activity and flux through the pathway of starch synthesis will be high. The importance of the triose phosphate transporter in regulating starch synthesis has been investigated in transgenic plants TPT activity reduced through expression of antisense RNA. In *Solanum tuberosum* leaves, TPT activity was reduced by 20-30% leading to an increase in leaf starch of 300% (Riesmeier *et al.*, 1993). In *Nicotiana tabacum* TPT activity was reduced to 80% yet leaf starch increased by only 10% (Barnes *et al.*, 1994). This difference between *Solanum tuberosum* and *Nicotiana tabacum* may be indicative of the involvement of factors other than the ratio of 3-PGA: $P_i$ that may vary between species.

Every step in an enzymic pathway contributes to some extent in the determination of flux (Kacser and Burns, 1973; Kacser *et al.*, 1995; ap Rees and Hill, 1994) and the effect of each enzyme on flux can be calculated as a flux control coefficient, which is the fractional change in flux resulting from a fractional change in enzyme activity. Some estimates of flux control coefficients have been made for *Arabidopsis thaliana* mutants deficient in chloroplastic phosphoglucomutase and AGPase and a *P. sativum* mutant deficient in branching enzyme. These values indicate that AGPase dominates

the control of starch synthesis in high and low light. However, flux control coefficients of PGM and SBE in high light were greater than in low light indicating that the balance of control may alter according to environmental conditions.
As more mutants are isolated and characterised (particularly in one species - see section 1.5) it should be possible to build up a fuller picture of the contribution of the enzymes involved starch synthesis to the control of the flux through the pathway.

### 1.2.3 Phosphorylation of Starch

In many species, amylopectin is phosphorylated - the phosphate group is monoesterified to O-6 or O-3 of a glucosyl unit. Phosphorylation occurs concurrently with starch synthesis at a frequency directly related to the chain length distribution of the amylopectin at levels ranging from one phosphate per 100 residues in *Solanum tuberosum*, to just a few per cent of this value in cereals. Blennow *et al.*, 1998 have demonstrated that degree of phosphorylation correlates with the chain length distribution of amylopectin. Moderately phosphorylated starches showed polymodal chain length distributions with main peaks at DP 14-15 and 50 whereas highly phosphorylated starches contain most chains with mean DP 19. As seen from Table 1.1, these chain length distributions are roughly those found in storage starch and transitory starch respectively.

Recently, a 155 kDa granule-bound protein was identified in *Solanum tuberosum* which is reported to influence both the level of phosphorylation of starch and the extent of its degradability (Lorbeth *et al.*, 1998). Reduction in levels of the protein (R1) throughout the plant by expression of antisense RNA resulted in a large decrease in phosphorylation of tuber starch. The level of starch in the leaves was higher than control plants and the net starch degradation in tubers was reduced. Expression of the gene in *E. coli*, however, led to a greater degree of phosphorylation of bacterial glycogen (Lorbeth *et al.*, 1998), suggesting it be directly involved with phosphorylation of glucan chains. This suggests that the amount of phosphorylation may be related to degradability of the starch.

### 1.3 Degradation of transitory starch

The release of fixed carbon from transitory starch is poorly understood. Elucidation of the mechanism and regulation of starch degradation is complicated by the existence of possible multiple pathways, multiple isoforms of degradative enzymes and the apparent constitutive expression of starch metabolic enzymes in photosynthetic tissues under normal conditions.

It is clear that starch can differ in its level of degradability. High molecular weight amylopectin and amylose in leaves may constitute a pool of material that is turned over at a slower rate than highly branched, short chain amylopectin. Transitory starch amylose molecules, in general, seem to be of lower molecular mass and contain more branches than storage starch amylose. The amylopectin contains shorter chains than those found in storage starch, and fewer intermediate and long chains than in storage amylopectin. This is consistent with the idea that a high concentration of non-reducing ends available for attack by degradative enzymes may enable fast-turnover transitory starch to be degraded more rapidly. It is unknown if it is the structure alone that determines whether it can be degraded rapidly or even if the pools might be degraded by different mechanisms.

Starch can be degraded by hydrolytic and phosphorolytic enzymes. These may represent two alternative pathways that *in vivo* might occur simultaneously or play different roles in starch metabolism. It has recently come to light that some degradative enzymes may have important roles in the synthesis of starch. This may be an indication that the processes of synthesis and degradation are not as distinct as at first thought in terms of the enzymes involved.

#### 1.3.1 Enzymes capable of hydrolytic degradation of transitory starch

**Amylases**

α-amylase (EC 3.2.1.1) is an endoamylase which catalyses hydrolysis of any α (1-4) linkages in starch except those immediately adjacent to α(1-6) linkages.

Multiple isoforms are present in the leaves of many plant species, and some isoforms are located in the chloroplast where they may be involved in the degradation of transitory starch. Native PAGE activity zymograms can used to gain qualitative

information about the number of isoforms of amylase present in crude extracts of leaves. From *Beta vulgaris*, five different forms were separated on zymograms, one extra-chloroplastic and four at least partly chloroplastic (Li *et al.*, 1992). From *Arabidopsis thaliana* four forms have been characterised: three endoamylases (at least one is chloroplastic; Zeeman *et al.*, 1998) and one extra-chloroplastic exoamylase (Lin *et al.*, 1988). The most recently characterised endoamylase is dark induced (Kakefuda and Preiss, 1997).

β-amylase (EC 3.2.1.2) is an exoamylase that cleaves maltose groups from the non-reducing end of α (1-4) glucan chains. Although previously thought to be located in the chloroplast, more recent studies show that β-amylase is located in the vacuole and hence it is unlikely to be involved in the degradation of transitory starch. In *Arabidopsis thaliana* more than 80% of the total amylolytic activity is that of β-amylase, and the enzyme is believed to be vacuolar and maybe located in the phloem (Lin *et al.*, 1988; Monroe *et al.*, 1991; Wang *et al.*, 1995).

**Debranching enzymes**

Debranching enzymes catalyse the hydrolysis of α (1-6) linkages and fall into two classes: isoamylases and pullulanases (also known as limit dextrinases or R enzymes). This distinction is made largely on the inability of isoamylase to hydrolase the α (1-6) linkages of yeast pullulan (a polysaccharide composed of maltotriose units linked end to end by α (1-6) linkages; Nakamura, 1996). Pullulanases have been shown to be present as chloroplastic and extra-chloroplastic isoforms in the leaves of a range of plants; isoamylases have been reported less, which may reflect the difficulties associated with measuring their activity in crude extracts. In *Arabidopsis thaliana* a pullulanase and a chloroplastic isoamylase have been identified (Zeeman *et al.*, 1998). A role for chloroplastic isoamylase in starch synthesis is covered under the section 1.2.2.2. An additional chloroplastic isoamylase sequence has been identified from the genome-sequencing project.

**α-glucosidase**

α-glucosidase (EC 3.2.1.20), also known as maltase, catalyses the hydrolysis of maltose and malto-oligosaccharides to glucose. Substrate preference can vary widely

between enzymes from different species, from specifically α (1-4) linkages and short malto-oligosaccharides to α (1-6) linkages and other glucosidic linkages in larger substrates. They may thus play a role at the forefront of starch degradation attacking large molecules, or act on the products of amylolytic digestion ensuring complete breakdown to glucose. A chloroplastic isoform has been studied in pea leaves (Sun *et al.*,1995).

### 1.3.2 Enzymes capable of phosphorolytic degradation of transitory starch

**Starch phosphorylase**

Starch phosphorylase (α-glucan phosphorylase, EC 2.4.1.1) catalyses the phosphorolytic cleavage of glucose from the non-reducing end of a glucan chain resulting in glucose-1-phosphate. Two distinct and highly conserved plastidial and extraplastidial isoforms (type I or L and type II or H) of phosphorylases have been identified in a number of plant species but studied most in *Solanum tuberosum*. Two plastidial isoforms denoted Phola and Pholb have been identified in *Solanum tuberosum* where they account for amyloplastic and chloroplastic activities respectively (Fukui *et al.*, 1987). *Pisum sativum* also contains two plastidial isoforms (Steup and Latzko, 1979).

**Maltose phosphorylase**

Maltose phosphorylase (EC 2.4.1.8) catalyses the inter-conversion of maltose with glucose-1-phosphate and glucose. Although the enzyme has been reported in *P. sativum* chloroplasts (Kruger and ap Rees, 1983), little else is known about its occurrence. It could potentially act on the products of amylolytic digestion ensuring complete breakdown to sugars.

### 1.3.3 The role of these enzymes in chloroplast starch degradation

In leaves, rapid biosynthesis and depletion of starch takes place. Net degradation occurs predominantly during the night although there may also be net degradation at the beginning and end of the photoperiod (for example in *Spinacia oleracea*, Servaites *et al.*, 1989; *Beta vulgaris* and *Phaseolus vulgaris*, Fondy *et al.*, 1989). It is generally believed that starch degradation in the chloroplast proceeds by phosphorolytic and hydrolytic pathways. *Spinacia oleracea* and *Arabidopsis thaliana* leaf chloroplasts contain endoamylase, debranching enzyme, phosphorylase, and D-

enzyme. In *Spinacia oleracea* α-amylase and phosphorylase constitute the major degradative activities in the chloroplast. In *Arabidopsis thaliana* most amylolytic activity and phosphorylase activity is found outside the chloroplast. Lin *et al.* (1988) found that the chloroplast contained 19% of total amylolytic activity, 4% total phosphorylase activity and almost all detectable D-enzyme activity. This led them to suggest that transglycosylation could be a significant breakdown pathway in *Arabidopsis thaliana*. Since all of these enzymes capable of degradative activity show constitutive expression, it is uncertain which are involved and what regulates the process. Although amylases have a sharp pH optimum around pH 6.0 (Beck and Ziegler, 1989) and some have been shown to be calcium-dependent (Briggs, 1967; Li *et al.*, 1992; Witt and Sauter, 1996), they have no properties that suggest how their activity could be modulated *in vivo*.

α-amylases (Steup *et al.,* 1983) and α-glucosidases (Sun and Henson, 1990) have been reported to be capable of attacking intact isolated starch granules. Conflicting results have been obtained on the ability of starch phosphorylase to do so (Kruger and ap Rees, 1983; Witt and Sauter, 1995; Steup *et al.*, 1983) which may be due to unphysiological circumstances in the *in vitro* degradation of isolated granules by purified enzymes. In *Arabidopsis thaliana* leaves, only one α-amylase has been shown to be dark-induced (the others are constitutively present). This enzyme may have a role in the initiation of starch granule breakdown (Kakefuda and Preiss, 1997).

Plastidial starch phosphorylase prefers malto-oligosaccharides to large branched glucans (Fukui *et al.,* 1987; Steup, 1988). The activity of starch phosphorylase is sensitive to pH and dependent on the concentration of $P_i$ and the enzyme is inhibited by ADPglucose. It is thus a possible point of regulation of starch degradation. In isolated chloroplasts, phosphorolytic starch degradation was shown to be sensitive to exogenous $P_i$ concentration both in *Pisum sativum* (Stitt and ap Rees, 1980; Kruger and ap Rees, 1983) and in *Spinacia oleracea* (Stitt and Heldt, 1981). $P_i$ concentration is a means of mediating a cytosolic demand for sucrose to control of starch degradation. However, removal of exogenous $P_i$ from isolated *Spinacia oleracea*

chloroplasts in the dark resulted in a shift from phosphorolytic to amylolytic degradation without altering the total rate of degradation (Stitt and Heldt, 1981). This indicates that there are other yet unidentified means of regulating the process.

Although in *Arabidopsis thaliana*, the plastidial starch phosphorylase activity is too low to account entirely for the rate of starch degradation in the dark, it could be involved together with D-enzyme, and may act to further digest the products of hydrolytic degradation by α-amylases. It may also have a role in starch synthesis, and the ratio of glucose-1-phosphate to $P_i$ may control the switch of activity in the direction of synthesis or degradation. Since D-enzyme catalyses a disproportionating reaction (see section 1.4.2) the net result of D-enzyme activity may be dependent on the available substrates and thus a role in both directions of degradation and synthesis may be possible for D-enzyme also. A role for debranching enzyme in starch synthesis has already been discussed (section 1.2.2.2) but since a number of isoforms have been identified a role in degradation may still be possible. It is clear that starch degradation involves co-ordinate activity between a number of enzymes and is closely regulated by mechanisms that have yet to be determined.

## 1.4 D-enzyme

### 1.4.1 Distribution of D-enzyme

D-enzyme, disproportionating enzyme, or 4-α-glucanotransferase (EC 2.4.1.25) was first found in tubers of *Solanum tuberosum* by Peat *et al.* (1956) but has also been found in roots of *Daucus carota* (Manners and Rowe, 1969), fruits of *Lycopersicon esculentum* (Manners and Rowe, 1969), germinated seeds of *Hordeum vulgare* (Yoshio *et al.*, 1986), tubers of *Ipomoea batatas* (Suganuma *et al.*, 1991) and leaves of *Spinacia oleracea* (Okita *et al.*, 1979), *Pisum sativum* (Kakefuda *et al.*, 1986) and *Arabidopsis thaliana* (Lin and Preiss, 1988).

### 1.4.2 The reaction catalysed by D-enzyme

D-enzyme catalyses glucan transfer in a "disproportionating" reaction, splitting off a maltosyl or larger group from one 1,4-α-D-glucan donor molecule and transferring

the group to another acceptor 1,4-α-D-glucan molecule by reforming the α-D-(1-4) bond. An equation to represent its most common action is

$$\alpha(1\text{-}4)\text{glucan}_{(a)} + \alpha(1\text{-}4)\text{glucan}_{(b)} \dashrightarrow \alpha(1\text{-}4)\text{glucan}_{(a+b)-1} + \text{glucose}$$

This reaction has been well-characterised *in vitro* using chromatographic techniques. In dextrins there are two 'forbidden' linkages that cannot be cleaved by D-enzyme – the bond that is penultimate to the reducing end and the bond at the non-reducing end. For this reason D-enzyme cannot cleave maltose and the smallest possible donor for the reaction is maltotriose where both forbidden linkages are the same bond. For the same reason maltose is not an acceptor nor is it ever produced, but maltosyl groups are transferred more rapidly between glucans than larger moieties. The rate of transfer of the maltosyl group to glucose is the same whether the donor is maltotriose, maltopentaose or maltohexaose (Jones and Whelan, 1969). The smallest acceptor molecule is D-glucose.

When allowed to proceed to equilibrium *in vitro* yields of products show that there are 'preferred' reactions caused by the availability of substrates after successive transfer reactions, the forbidden linkages and the favoured transference of a maltosyl group. Suganuma *et al.* (1991) have proposed a possible subsite structure for D-enzyme by studying the products of D-enzyme in an amylase-free *Ipomoea batatas* variety. For example for maltotriose: the first G3 molecule binds to the active site of the enzyme in such a way that the second (reducing end) glycosidic bond is at the catalytic site. This bond is cleaved with the formation of a maltosyl-enzyme complex and the release of glucose. The second molecule of G3 binds to the complex as an acceptor to form a new α-1,4-linkage at its non-reducing end producing G5. In this case the substrates available for the next reaction are G1 and G5 which give rise to more G5 or G3. When higher malto-oligosaccharides were provided, the sizes of the major product increased correspondingly but this decreased when the reaction was allowed to proceed to equilibrium.

Amylose and amylopectin can function as both donors and acceptors for the action of D-enzyme, and even long α-1,4-glucan units (Takaha *et al.*, 1996) or highly

branched cluster units of amylopectin (Takaha, 1996) can be transferred between molecules.

### 1.4.3 The production of novel cyclic molecules by potato D-enzyme

Amylose and amylopectin can also act as both donor and acceptor at the same time in intramolecular transglycosylation reactions to produce cyclic α (1-4)-glucans (cycloamylose) (Takaha *et al.*, 1996) and cyclic α (1-4),α (1-6)-glucans (Takaha *et al.*, 1998). Potato D-enzyme purified from recombinant *E. coli* acting on synthetic amylose produced cyclic molecules ranging in size from 17 DP to several hundred. D-enzyme did not act upon purified cycloamylose, but if glucose was added as an acceptor molecule, smaller cyclic and linear molecules were produced. Crystallographic analysis shows that cycloamylose adopts an antiparallel helix structure – two left-handed single helices connect forming a "figure eight" when viewed end-on (Gessler *et al.*, 1999). Cycloamylose was also reported to be produced by 4-α-glucanotransferase purified from *Thermococcus litoralis* (Jeon *et al.*, 1997). These cyclic glucans have not yet been detected *in vivo*, although it is possible that they may have a short half-life.

### 1.4.4 Localisation and expression of D-enzyme

The subcellular localisation of D-enzyme in leaves has been studied for several species. D-enzyme activity was found in both chloroplastic and cytosolic fractions in *Pisum sativum* (Kakefuda *et al.*, 1986) and in *Spinacia oleracea* (Okita *et al.*, 1979). In *Arabidopsis thaliana* leaves (Lin and Preiss, 1988) the activity was confined to the chloroplastic fraction.

The cDNA for D-enzyme from potato tubers encodes a putative plastid transit peptide, which is thought to target the protein to both amyloplasts and chloroplasts. It is well established that transit peptides can function interchangeably between plastids (de-Boer *et al.*, 1988; Klosgen and Weil, 1991).

In potato, D-enzyme mRNA is present in leaves, stems, roots and stolons and is most abundant in developing and mature tubers. The transcript also increases in response to light and to the addition of sucrose to the medium suggesting that it accumulates

under circumstances when starch biosynthesis is most active. Transcript declines in amount where starch is broken down (cold-stored tubers, sprouting tubers and dark incubated leaves) but protein expression remains high (Takaha, 1996).

### 1.4.5 The physiological role of D-enzyme

#### 1.4.5.1 D-enzyme (amylomaltase) in micro-organisms

Understanding of the *in vivo* role of 4-α-glucanotransferases is most advanced in microorganisms where a large number of related genes have been isolated (for review see Takaha and Smith, 1999). Potato D-enzyme has up to 40.2% amino acid sequence identity with bacterial amylomaltases (Takaha, 1996). Bacterial amylomaltase gene expression is controlled as part of an operon. Since genes on the same operon are co-ordinately regulated this has been taken to suggest co-ordinate activity between the gene products. In *E. coli*, amylomaltase is part of a malto-oligosaccharide transport and utilisation system, which includes maltodextrin phosphorylase and maltose transport proteins (Schwartz, 1987). The role of amylomaltase is apparently to lengthen short malto-oligosaccharides so that they can be degraded by glucan phosphorylase. Like plant starch phosphorylases, this enzyme is unable to degrade chains shorter than five residues. The genes for amylomaltase and glucan phosphorylase together constitute the *MalPQ* operon. However, in some other bacterial genomes (*H. influenzae*, Fleischmann *et al.*, 1995; *A. aeolicus*, Deckert *et al.*, 1998) amylomaltase is part of the glycogen operon which includes genes of glycogen synthesis and degradation. These organisms also do not contain the genes homologous to those necessary for malto-oligosaccharide transport into the cytoplasm of *E. coli*. These observations suggest amylomaltase in these organisms seems more likely to be involved in glycogen metabolism than exogenous malto-oligosaccharide utilisation.

#### 1.4.5.2 D-enzyme in plant starch metabolism

In plants, the long-held view of the function of D-enzyme is to convert short malto-oligosaccharides into larger ones which serve as substrates for breakdown by starch phosphorylase (Lee and Whelan, 1971; Lin and Preiss, 1988) in a system analogous to that of *E. coli*. However, this view is speculative and remains unsupported by

experimental evidence. Other speculative ideas for roles have been suggested on the basis of *in vitro* studies and current hypotheses regarding granule synthesis. Takaha (1996) suggests that cyclic glucans might be generated by D-enzyme from amylose and amylopectin as an intermediate in starch breakdown. Starch could even be transported as cyclic glucan – this process would be more energetically favourable than pathways involving sucrose for transfer to the sink organ. This hypothesis would also help explain the existence of amylases and phosphorylases in the cytosol fraction (Beck and Ziegler, 1989) and the phloem specific localisation of β-amylase in *Arabidopsis thaliana* plants (Wang, *et al.* 1995). In bacteria, a cyclic glucan specific transporter system has been reported which involves cyclic α (1-4) glucan-forming enzyme and cyclic α (1-4) glucan hydrolases (Fiedler *et al.*, 1996).

D-enzyme has potential roles in amylopectin synthesis. Firstly, it could transfer malto-oligosaccharides between side chains in the construction of amylopectin clusters. Secondly, it could transfer clusters from place to place within the amylopectin molecule or from a soluble pre-amylopectin intermediate to the amylopectin molecule elongating along the surface of the granule. Thirdly, it may be involved in recycling malto-oligosaccharides produced by the 'trimming' activity of debranching enzyme on the elongating amylopectin molecule according to the 'pre-amylopectin trimming' model (Ball *et al.*, 1996). Finally, it might play a scavenging role, recycling malto-oligosaccharides and/or soluble glucans produced by the action of starch synthase and SBE on malto-oligosaccharides (Zeeman *et al.*, 1998) ensuring synthetic activity is directed to the granule. In the latter two activities, starch phosphorylase could be also involved - this would explain the induction of D-enzyme and starch phosphorylase genes during starch synthesis (St.Pierre and Brisson, 1995) and could also explain the substrate preference of plastidic starch phosphorylase for malto-oligosaccharides.

Studies of transgenic and mutant organisms have not yet clarified the physiological role of D-enzyme. D-enzyme activity in potato plants was reduced to 1% of wild-type activity by transformation with sense and antisense copies of the potato D-enzyme gene (Takaha *et al.*, 1998). The lack of D-enzyme resulted in slow plant

growth, but development was apparently normal. However, stored transgenic tubers show a delayed sprouting phenotype suggesting that D-enzyme may have a role in mobilising starch for growth (Takaha, 1996). Starch content of tubers was not appreciably altered in amount, proportion of amylose, molecular weight of debranched amylopectin, or branch chain length. The residual 1% of wild-type activity could be just sufficient to fulfil its role so that starch appears normal.

Recently, a D-enzyme mutant was isolated from *Chlamydomonas reinhardtii*, a starch-storing unicellular alga. *C.reinhardtii* accumulate a polysaccharide similar to cereal endosperm storage starch when grown under nutrient starvation conditions. Transitory starch synthesis is obtained under conditions of active photosynthesis and cell division. The *sta11-1* mutants show a lack of D-enzyme that correlates with an abnormal accumulation of malto-oligosaccharides, large decreases in total starch and an increase in the proportion of amylose. Colleoni *et al.* (1999a) explain these observations as a requirement for D-enzyme in normal starch biosynthesis. Colleoni *et al.* (1999b) attempt to explain their results in terms of the pre-amylopectin trimming model: namely that D-enzyme uses malto-oligosaccharides (derived from debranching activity) as donors to yield net incorporation into amylopectin at the surface of the granule. *In vitro* incorporation of radiolabelled malto-oligosaccharides into amylopectin demonstrated that D-enzyme was capable of this activity. However, it is unclear from where these malto-oligosaccharides are derived. This model assumes the malto-oligosaccharides result from the action of debranching enzyme yet there are conflicting interpretations of results derived from debranching enzyme mutants of *C.reinhardtii* (Mouille *et al.,* 1996) and *Arabidopsis thaliana* (Zeeman *et al.,* 1998). The scavenging role for debranching enzyme explains why phytoglycogen and starch accumulate simultaneously. The *sta11-1* mutants also accumulated a significant amount of water-soluble, amyloglucosidase digestible polysaccharide. This is presumably produced by the action of starch synthase and SBE on the malto-oligosaccharides. In the presence of abnormal accumulation of malto-oligosaccharides it would be expected that considerably more phytoglycogen would be synthesised. There may be insufficient debranching enzyme activity to degrade all of this material. The presence of this material would thus be consistent with the

scavenging model. The presence of the elevated levels of malto-oligosaccharides are also consistent with a role for D-enzyme in the degradation of malto-oligosaccharides by starch phosphorylase and D-enzyme in order to recycle malto-oligosaccharides produced during synthesis. Stimulation of degradation by phosphorylase in the presence of D-enzyme was observed *in vitro*. The increase in the proportion of amylose could be attributable to stimulation of amylose synthesis by the high concentrations of malto-oligosaccharides. This has been reported in *P. sativum* (Denyer *et al.*, 1997).

The extent to which the mutant phenotype in *C.reinhardtii* can be interpreted in terms of higher plant starch metabolism is uncertain. Colleoni *et al.* (1999b) define the *sta11-1* mutation as partly conditional – it is expressed in both growth conditions but maximal expression of the defect is obtained in nitrogen starvation conditions (when the cell devotes its activity to carbohydrate storage and the energy supply remains limited) where 'decreases in starch amounts exceeding 90%' are obtained. No explanation is suggested as to why total starch should decrease to this extent even though other starch synthetic enzymes exhibited normal activities. This 'decreased starch' phenotype was not recorded under the growth conditions where *C.reinhardtii* synthesises transitory starch. No growth defects were detected in the mutant when grown under varying light and growth conditions. Caution must be exercised in the interpretation of what might be the result of complex pleiotropic effects.

The results obtained from the *C.reinhardtii* mutant provide some valuable information as to the *in vivo* role of D-enzyme. However, they should be considered in a wider context than that provided by the 'pre-amylopectin trimming' model since no experimental evidence substantiates this view. Production and characterisation of mutants defective in D-enzyme and one or more other starch metabolic enzymes may help to clarify its role further.

### 1.5 Molecular dissection of starch metabolism through collections of mutants

Mutants deficient in various enzymes have already contributed much to current understanding of starch metabolism. Some mutations result in clear phenotypes (for

example in that of Mendel's wrinkled peas) others (for example excess or reduced starch) phenotypes for which it is necessary to devise screens of mutagenized populations. Progress in transformation has enabled more direct approaches, but requires gene sequence information.

Recently attention has turned towards isolating starch mutants in model systems rather than crop species, with a view to obtaining a whole battery of mutants with which to dissect the pathways involved. A collection of starch metabolic mutants in *Chlamydomonas reinhardtii* is being assembled by the laboratory of Steven Ball. Mutants isolated include plants lacking ADP-glucose pyrophosphorylase, soluble starch synthase I and II, granule bound starch synthase I, debranching enzyme and most recently, D-enzyme. Double and triple mutants, produced by crossing mutants for debranching enzyme with mutants for granule bound starch synthase I and starch synthase II (Dauvilleé *et al.,* 1999) were used to confirm that the debranching enzyme mutant of *C.reinhardtii* synthesises phytoglycogen instead of starch (the debranching enzyme mutant of *Arabidopsis thaliana* synthesises starch and phytoglycogen simultaneously, section 1.2.2.2).

*Arabidopsis thaliana* too has become a valuable model system for studying the processes and importance of starch metabolism. Mutant populations have been generated by x-ray mutagenesis and ethyl methanesulphonate (EMS) mutagenesis. Mutants have been isolated from such populations by phenotypic screen, typically by screening for alterations in the structure of leaf starch by iodine staining. Most of the starch mutants in *Arabidopsis thaliana* have been obtained using this (or variations on this) method. Mutants obtained to date are summarised in Table 1.2.

**Table 1.2 *Arabidopsis* mutants deficient in starch metabolic enzymes**

| STARCH MUTANT | REFERENCE | SCREENING METHOD | PHENOTYPE ( in screen) |
|---|---|---|---|
| chloroplastic phosphoglucomutase | TC7 Caspar *et al.*, 1985 | iodine stain screen | no starch |
| ADPglucose pyrophosphorylase | *adg1*/TL24,TL25 Lin *et al.*, 1988 *adg2* /TL46,TL3 Lin *et al.*,1988 | iodine stain screen | no starch |
| chloroplastic isoamylase | *dbe1*/ SZ20 Zeeman *et al.*, 1998b | iodine stain screen at end of night | altered starch |
| chloroplastic endoamylase | SZ63 Zeeman *et al.*, 1998a | iodine stain screen | high starch |
| regulation genes of β-amylase | stf1; sop1(see *sex*) Caspar *et al.*, 1989 | iodine stain screen | no starch or high starch |
| hexose transporter | TC265 Trethewey and ap Rees,1994 | iodine stain screen at end of night | high starch |
| unknown starch regulatory genes | *sex1*/TC26 *sex2*/TL50 *sex3*/TL54 Caspar *et al.*, 1991 | iodine stain screen | high starch |
| unknown starch regulatory gene | cam1 Eimert *et al.*,1995 | iodine stain screen | high starch at the onset of flowering |

Not only is *Arabidopsis thaliana* a directly relevant model for starch metabolism in vascular plants, but it can be readily and stably transformed. This enables the production of specific mutants by homologous recombination (for example, using site directed mutagenesis to change specific residues), and the production of populations of mutant plants containing transposon or T-DNA insertions from which specific mutants can be isolated by sequence-based screening (this is discussed in Chapter 4). It also allows genes to be reintroduced into mutant plants in order to complement mutant phenotypes and the reintroduction of genes under the control of inducible promoters. The T-DNA and transposon tagged populations together with the genome sequencing project and EST database should facilitate isolation of

mutants for any starch metabolic enzyme obtained in other species (provided sequence information can be obtained), regardless of phenotype.

### 1.6 Aim of the project

Despite extensive biochemical characterisation of the activity of D-enzyme *in vitro*, few conclusions have been reached about the role of the enzyme in starch metabolism *in vivo*. No mutants have been obtained in any higher plants, and transgenic potato plants with D-enzyme activity reduced to 1% by expression of antisense genes showed no detectable alteration in the amount or structure of starch in their tubers. The localisation and expression patterns of D-enzyme in the plant, and its *in vitro* activity strongly suggest that the enzyme does have an important role in starch metabolism despite this result. Considerable gaps remain in the understanding of granule synthesis and particularly starch degradation and determining the role of D-enzyme might provide important clues to help complete this picture. Since it was possible that the residual 1% of wild-type activity could be sufficient for starch metabolism to appear normal, it was of interest to obtain a plant completely lacking D-enzyme.

The initial aims of this project were to isolate and characterise the gene in *Arabidopsis thaliana*. The *Arabidopsis thaliana* D-enzyme gene was to be expressed in an *E. coli* expression vector to produce protein in order to prepare antibodies from recombinant protein. Using reverse genetics, the D-enzyme gene sequence would be used to screen populations of T-DNA tagged populations of *Arabidopsis thaliana* in order to isolate a plant containing an insertion that disrupts the D-enzyme gene. After confirming the plant was null mutant for D-enzyme activity, it could then be studied for changes in growth and development, and analysed to determine the effects of lack of D-enzyme, in particular, on starch accumulation, composition and structure. Such a mutant could also be used in such future work as preparing plants with D-enzyme under the control of an inducible promoter, and preparing plants which contain mutations in both the D-enzyme gene and another gene of starch metabolism, for example starch phosphorylase or starch debranching enzyme. The D-enzyme null mutant plant would thus be a valuable tool in the investigation of the *in vivo* role of D-enzyme in starch metabolism.

# Chapter 2

# Materials and Methods

## 2.1 Biological materials

### 2.1.1 Plant material

*Arabidopsis thaliana* (L.) cv. Columbia (Col) seed was obtained from the Nottingham *Arabidopsis* Stock Centre.

T-DNA transformed *Arabidopsis thaliana* (L.) cv. Wasserilewskija (Ws) populations were obtained from:

Feldmann/Dupont collection (Feldmann 1991): Dupont

Versailles collection (Bechtold *et al.*, 1993): Uni Paris, Orsay (courtesy of Professor Martin Kreis).

Plants were grown on Levington F2 compost : vermiculite : sand (3:1:1) in a growth cabinet at 20°C with a 10h photoperiod at an irradiance of 20 μmol $m^{-2}$ $s^{-1}$ photosynthetically active quanta.

### 2.1.2 Bacterial strains and genotypes

| ***Escherichia coli*** **strain and source:** | **Genotype:** | **Use:** |
|---|---|---|
| XL1-Blue | *sup*E44 *hsd*R17 *recA*1 *gyrA*46 *thi relA*1 *lac*⁻ F' [*proAB*⁺ *lacI*q *lac*ZΔM15 Tn10 (*tet*$^r$)] | host for recombinant manipulation |
| JM101, Promega. | *sup*E *thi* Δ(*lac-pro*AB) [F' *tra*D36 *pro*AB *lac*I$_q$Z ΔM15] | host for recombinant manipulation |
| LE392, Promega. | *hsd* R574 ($r_{K-}m_{K+}$ *sup*E44 *sup*F58 *lac*Y1 or (*lac*IZY)6 *gal*K2 *gal*T22 *met*B1 *trp*R55 | host for λGEM11 |
| Y1090(ZL), Gibco-BRL. | Δ*lac*U169 *pro*A⁺ *hsd*R⁻ *hsd*M⁺ Δ*lon ara*D139 *str*A *sup*F (*trp*C22::Tn10) | host for λZIPLOX™ libraries |
| DH10B(ZIP), Gibco-BRL. | F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *end*A1 *rec*A1 *deo*R Δ(*ara, leu*)7697*ara*D139 *gal*U *gal*K *nup*G *rps*L λ*xis*6 *ind*⁻ pZIP1 (pZIP1 = P1 *ori*-kanR⁻ *cre*) | host for pZL1 (resulting from cDNA clone excision from λZIPLOX™) |

### 2.1.3 Bacterial plasmids and bacteriophage

| Vector | Source | Use |
|---|---|---|
| pBluescript II SK +/- | Stratagene | Subcloning |
| pTrc99A | Promega | Protein expression vector |
| λ Ziplox™ | Gibco-BRL | cDNA library vector |
| λGEM11 | Promega | Genomic library vector |

### 2.1.4 Potato D-enzyme cDNA clone

The pBluescript II $SK^+$ plasmid carrying a 2.1 kb *Not I* cDNA fragment encoding potato D-enzyme is referred to as pDPE102M and was a gift from T. Takaha, Ezaki Glico Co. Ltd., Japan.

### 2.1.5 Potato plastidic starch phosphorylase cDNA clone

The pUC19 plasmid carrying a 0.3 kb *Eco RI* cDNA fragment, which contains 22 bases of the 5'noncoding region and the coding region corresponding to amino acids –50-38 (Nakano *et al.*, 1989) is referred to as pUC-PSP and was a gift from T.Fukui, Osaka University, Japan.

## 2.2 Miscellaneous

### 2.2.1 Chemicals

Analytical grade chemicals and reagents were purchased from BDH Chemicals Ltd., Sigma Chemicals Co. Ltd. or Fisher Scientific UK.

### 2.2.2 Radiochemicals

[$\alpha$-$^{32}$P]dCTP (3000 Ci $mmol^{-1}$) was purchased from Amersham International plc.

### 2.2.3 Autoradiography film

X-ray films were Cronex-4 (Sterling Diagnostic Imaging Inc.) and all films were developed in a Compact X2 (X-Ograph, Ltd.) automatic developing machine.

### 2.2.4 Bacteriological media

| | |
|---|---|
| Luria-Bertani medium (litre$^{-1}$): (L-broth) | 10 g bacto-tryptone (Difco laboratories),<br>5 g bacto-yeast extract (Difco laboratories),<br>10 g NaCl, pH 7.0. |
| LB agar (litre$^{-1}$): | As for LB medium with the addition of<br>15 g bacto-agar. |
| BBL top agar (litre$^{-1}$): | 10 g BBL trypticase (Difco laboratories),<br>5 g NaCl,<br>6.5 g bacto-agar. |
| Phage buffer (litre$^{-1}$): | 7 g $Na_2HPO_4$,<br>3 g $KH_2PO_4$ (anhydrous, AnalaR),<br>5 g NaCl,<br>10 ml 100 mM $MgCl_2$,<br>10 ml 100 mM $CaCl_2$,<br>1 ml 1% (w/v) gelatine. |
| Terrific broth (litre$^{-1}$): | 12 g bacto-tryptone,<br>24 g bacto-yeast extract,<br>100 ml 100 mM $KPO_4$,<br>4 ml glycerol. |
| 2×YT (litre$^{-1}$): | 16 g bacto-tryptone,<br>10 g bacto-yeast extract,<br>5 g NaCl, pH 7.4. |

### 2.2.5 Plant sterile culture media

| | |
|---|---|
| MS medium (litre$^{-1}$): (Murashige and Skoog, 1962) | 4.4 g MS salts (Sigma),<br>20 g sucrose,<br>6 g bacto-agar, pH 5.6. |
| Gamborg medium (litre$^{-1}$): (Gamborg *et al.*, 1968) | 3.8 g B5 medium (Imperial Laboratories)<br>20.7 g sucrose<br>5 g MES, pH 5.7<br>9 g bacto-agar |

### 2.2.6 Restriction endonucleases and DNA modification enzymes

Restriction endonucleases and DNA modification enzymes were purchased from Boehringer Mannheim Biochemicals, Promega Corporation, Gibco-BRL Life Technologies, Inc., and New England Biolabs (UK) Ltd.

### 2.2.7 Materials used for SEM analysis of starch granules

Stubs, gold coating and other materials were all obtained from Agar Scientific, and ISS Group.

## 2.3 DNA isolation, manipulation and analysis

### 2.3.1 Small scale plasmid preparation

1.5 ml overnight culture was used to harvest *E. coli* cells for DNA extraction.

For checking clones, digests, transformations etc. a quick, crude method of DNA extraction was used. Cells were resuspended in 150 µl STEL buffer (8% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 0.5 mg $ml^{-1}$ lysozyme), boiled for 30 sec, then centrifuged for 10-20 min at top speed in a microfuge. The pellet was removed with a sterile wooden pick and the DNA in the supernatant precipitated by the addition of an equal volume of isopropanol. The DNA pellet was collected by centrifugation, washed in 70 % (v/v) ethanol and air-dried for 5 min. DNA was dissolved in 50 µl $dH_20$ or TE.

High quality DNA for purposes such as sequencing was prepared using Wizard® SV miniprep kit (Promega) or Qiaprep Spin Miniprep Kit (Qiagen).

### 2.3.2 Extraction of DNA from gels and PCRs

DNA fragments were extracted from gels and purified from PCR mixtures using QIAEX II Gel Extraction Kit, QIAquick Gel Extraction Kit or QIAquick PCR Purification Kit (all from Qiagen).

### 2.3.3 Isolation of bacteriophage λ DNA

200 µl fresh overnight culture of *E. coli* LE392 was infected with $10^8$ plaque forming units for 30 min at 37°C then added to a 250 ml flask containing 50 ml L-broth with 10 mM $MgSO_4$ and 0.1% (w/v) glucose. The culture was incubated overnight with shaking at 37° C then 50 µl chloroform was added and the mixture incubated at 4°C

for 2h to ensure total lysis of cells. Cultures were transferred to 50 ml Falcon tubes and centrifuged 6000 rpm 15 min to pellet debris. 3 ml culture supernatent was transferred to microfuge tubes to prepare DNA for initial analysis by the method of Waites (1995) and the remainder stored at 4°C until DNA was prepared using Qiagen Midi Kit (Qiagen) to use for subcloning.

### 2.3.4 Small scale isolation of *Arabidopsis* DNA

Three leaves were ground in 900 µl extraction buffer (100 mM sodium diethyldithiocarbamate, 100 mM EDTA pH 8.0, 3× SSC, 2% (w/v) SDS) with a glass pestle and tube until completely homogenous. 1 ml was transferred to a microfuge tube and vortexed for 30 sec with 0.5 ml chloroform. The mixture was centrifuged for 5 min at top speed, and the supernatant removed to a new tube. An equal volume of 1:1 phenol:chloroform was added and the tube vortexed and centrifuged as before. DNA was precipitated from the supernatant with an equal volume of ethanol and collected by centrifugation. The pellet was dissolved in 100 µl TE containing 20 µg $\mu l^{-1}$ RNase A. After 1h all soluble material was transferred to a clean tube containing 14µl 5M NaCl. After mixing, 114µl 2% (w/v) CTAB solution was added and the tube mixed by inversion. The DNA was again pelleted by centrifugation and the CTAB solution removed. The pellet was washed overnight in 1 ml 70 % (v/v) ethanol, 150 mM NaCl, then collected by centrifugation and dissolved in 50 µl TE.

### 2.3.5 DNA sequencing

DNA sequencing was performed using a dRhodamine terminator cycle sequencing kit and the products were separated on an ABI Prism 377 XL PCR machine (PE Applied Biosystems).

### 2.3.6 Labelling of double-stranded DNA fragment

Labelling of double-stranded DNA fragments with [$\alpha$-$^{32}$P]dCTP was carried out by the random primer method (Feinberg and Vogelstein, 1983). The DNA fragment (30-50 ng in 34 µl) was heat denatured at 100°C for 3 min then chilled on ice. To the denatured DNA, 10 µl of 5× OLB buffer (250 mM Tris-HCl (pH 8.0), 25 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 2 mM ATP, 2 mM dTTP, 2 mM dGTP, 1M HEPES-NaOH (pH 6.6), 1 mg $ml^{-1}$ random hexadeoxyribonucleotides), 2 µl of 10mg

ml $^{-1}$ BSA, 3 µl of [α-$^{32}$P]dCTP (30µCi) and 1 µl (1 unit) of DNA polymerase I-Klenow fragment were added. The reaction was allowed to proceed for 1h at 37°C, then was applied to a Sephadex G-50 column (made using a 1 ml syringe barrel) to remove unincorporated nucleotides. Labelled DNA was collected in 400 µl of TE buffer.

### 2.3.7 D-enzyme primers for sequencing and other DNA manipulations except those used for screening of T-DNA lines

| Oligo name | Location/cDNA | $t_m$°C / GC% | Primer sequence (5'→3') |
|---|---|---|---|
| 5'END | -48 | 69.8°C / 56% | CTGGTGGATTAGAGTGGAGTGGAGC |
| 49-3' | 292 | 70.5°C / 54% | GAAGAGCTGGCGTTTTGCTACACC |
| 52-3' | 1221 | 71°C / 54% | CATGGTTGGACGATGGAAGGTAGG |
| 53-3' | 1583 | 71.2°C / 50% | CATTCTCTTCAACCGCTCAAACCG |
| 467-5' | 467 | 70.5°C / 50% | CAAGCCGTCTTTCACAAGCTCATC |
| 312-5' | 1023 | 55.6°C / 46% | ACACCGCTAACAAGAAGAGGAAAG |
| 35-5' | 210 | 69.5°C / 54% | CGGTAGCCACTGCTCATATTCTGC |
| 3'END | 1906 | 67°C / 40% | CGATCCACTATTTCAAACAACATGC |

The DPE primers presented in the above table were synthesised by Genosys Biotechnologies (Europe) Ltd., or Oswell DNA Service. The annealing position of these primers within the D-enzyme cDNA is illustrated in Figure 2.1.

## 2.4 Screening of libraries

### 2.4.1 Screening of *Arabidopsis* cDNA library

The λPRL2 cDNA library (Newman *et al.*, 1994) containing *Arabidopsis thaliana* (L.) cv. Columbia wild-type cDNA from all developmental stages was plated on *E. coli* Y1090(ZL) cells and transferred to Hybond-3C nitrocellulose filters (Amersham). The filters were hybridised with a $^{32}$P-labelled full length potato D-enzyme cDNA probe (obtained by digestion of pDPE102M) in buffer containing 6× SSC, 5× Denhardt's Solution (Sambrook *et al.*, 1989), 1% (w/v) SDS and 100 µg

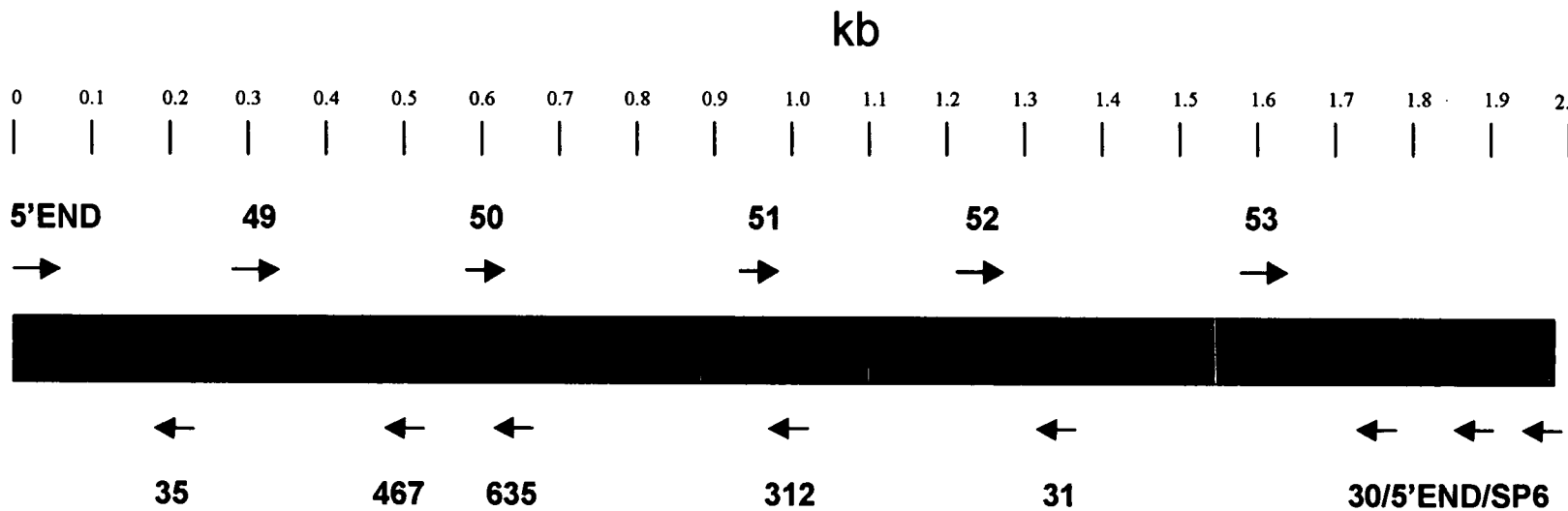



**Figure 2.1 Sequencing D-enzyme cDNA: primer annealing positions**

ml $^{-1}$ denatured herring sperm DNA, at 55°C for 18 h. $^{32}$P-DNA probes were prepared using a random oligonucleotide primer method (Feinberg and Vogelstein, 1983). Filters were washed in 6× SSC, 0.1% (w/v) SDS, 2× SSC, 0.1% (w/v) SDS and 1× SSC, 0.1% (w/v) SDS for 15 min at 55°C and then autoradiographed.

The λPRL2 cDNA library was constructed using λZIPLOX™ phage enabling cDNA to be recovered in autonomously replicating plasmid pZL1 using a simple *in vivo* excision protocol. λZIPLOX™ phage (which harbours cis-acting locus - *lox*P) were used to infect *E. coli* DH10B (harbouring the trans-acting *cre* gene on the resident plasmid pZIP) and D-enzyme cDNA clones in pZL1 were excised and re-circularised from λZIPLOX™ by Cre-*lox*P recombination.

### 2.4.2 Sequencing of D-enzyme partial clone

Two independent clones obtained from the λPRL2 library screen were sequenced by the dideoxy chain termination method of Sanger *et al.* (1977).

### 2.4.3 Screening of *Arabidopsis* genomic library

A genomic library (made in the laboratory of Elliot M. Meyerowitz, California Institute of Technology - gift of Justin Goodrich, University of Edinburgh) containing DNA from *Arabidopsis thaliana* (L.) cv. Columbia was plated onto *E. coli* LE392 cells and transferred onto Hybond-3C gridded membranes (Amersham). The filters were hybridised with 1.3 kb *Arabidopsis* D-enzyme cDNA sequence prepared by PCR and $^{32}$P-labelled as before, in 6× SSC, 5× Denhardt's Solution, 1% (w/v) SDS and 100 μg ml$^{-1}$ herring sperm DNA at 65°C for 18 h. Filters were washed twice for 15 min in 2× SSC, 0.1% (w/v) SDS, at 65°C then subject to autoradiography. Positively hybridising plaques were bulked in order to prepare λ DNA.

## 2.5 Construction of an *Arabidopsis* cDNA library enriched for sequences from genes of starch metabolism

### 2.5.1 Isolation of total RNA

7g *Arabidopsis* leaves were harvested and soaked petiole down in 8% (w/v) sucrose solution under light conditions for 24 h. This material was then frozen in liquid

nitrogen and then stored at -80°C. Frozen tissue was homogenised in a metal beaker under liquid nitrogen and divided between two 50 ml Falcon polypropylene tubes. Tubes were placed at -80°C until the liquid Nitrogen had evaporated, then 20 ml chilled fresh extraction buffer (100 mM Tris-HCl (pH 8.5), 10 % (w/v) TNS (Kodak) and 6 % (w/v) 4-aminosalicylic acid) was added and the tissue powder homogenised for a further 30 seconds. An equal volume of phenol:chloroform: isoamyl alcohol (25:24:1 (v/v) phenol saturated with TE) was added immediately, the homogenates mixed thoroughly by vigorous shaking, and then centrifuged at 5*g* for 20 min at 4°C. The upper aqueous phase was transferred to new tubes and precipitated with one-tenth volume of 3 M sodium acetate and 2.5 volumes of ethanol (-20°C) for 2 h. The precipitate was collected by centrifugation and dissolved in 4 ml DEPC-treated water. RNA was selectively precipitated by addition of an equal volume of 5 M LiCl, and incubated at 4°C overnight. Precipitated RNA was collected by centrifugation at 8*g* for 10 min at 4°C, dissolved in DEPC-treated water and ethanol precipitated (with one-tenth volume 3 M sodium acetate and 2.5 volumes of ethanol) to remove salt and finally washed in 70 % (v/v) ethanol. The RNA pellet was then redissolved in 1 ml of TE.

### 2.5.2 mRNA purification

Pharmacia Biotech mRNA purification kit was used to prepare polyadenylated RNA from "total" RNA using oligo (dT)-cellulose spin columns (centrifugable ion exchange columns to which the RNA is bound, washed and eluted by a change in salt concentration). Polyadenylated RNA was concentrated by ethanol precipitation with 20μg molecular biology grade glycogen.

### 2.5.3 cDNA synthesis and λ cloning

5 μg mRNA was used to make first strand cDNA as described in the Gibco BRL Superscript ™ Lambda System for cDNA synthesis and λCloning (Cat.Series 18256) instruction manual. This system was followed until the point of size fractionation chromatography, which was carried out using Pharmacia S-400 columns. 20-40 ng dried cDNA > 400 bps was then ligated into λ Ziplox™ *Not I-Sal I* arms (Gibco BRL) as described in the instruction manual. The ligated vector-cDNA was packaged into λ-phage using Gigapack®II Plus Packaging Extracts (Stratagene) and then plated on

*E. coli* Y1090(ZL) (Gibco BRL) for titering and amplification. Titer of the library prior to amplification was 200 000 plaque-forming units. This library was screened for a full length D-enzyme clone using a homologous probe (described in section 3.2.1), but no longer clones were identified. The library was also screened for starch phosphorylase clones using potato starch phosphorylase as a probe but this is not described in this study further than to mention the isolation of two clones.

## 2.6 Screening of T-DNA lines

### 2.6.1 DNA from pools of transgenic plant lines

DNA from pools of T-DNA-transformed plant lines was prepared as described in Krysan *et al.* (1996) by Sarah Sherson, Veronique Germain and Susan Forbes (University of Edinburgh). DNA was prepared from pools of 100 plant lines. Superpools of 700 plant lines were prepared by mixing equal amounts of DNA from 7 pools of 100 lines each and Megapools (of ~3500 plant lines) from mixing equal amounts of DNA from 5 Superpools.

### 2.6.2 DNA from 'subpools' of transgenic plant lines

Seeds from Dupont pool 2400 were obtained from Dupont.

Seeds were surface sterilised (15 min 5 % (v/v) sodium hypochlorite containing 200 $\mu l\ l^{-1}$ Triton X-100), rinsed four times with sterilised distilled water and plated in 0.1% (w/v) agarose onto 0.8 % (w/v) agar- Gamborg medium containing 50 $\mu g\ ml^{-1}$ kanamycin. Following a 24 h cold treatment (4°C to synchronise germination) the plates were transferred to continuous fluorescent light and grown for 1 week. Kanamycin-resistant seedlings were transplanted onto soil and grown in the greenhouse. One leaf was removed from each two-week-old plant and placed in a packet together with the other 59 from the same tray. Twelve packets of 60 leaves (90-180 mg tissue) were frozen in liquid nitrogen and stored at –70°C. The contents of each packet was ground to a fine powder in a chilled mortar and transferred to a 1.5 ml microfuge tube. DNA was prepared from the leaf powder using DNeasy Plant Mini Kit (Qiagen). After PCR two trays giving a positive result were selected. One leaf was removed from each plant in each tray and placed in a packet containing

leaves from two rows (20 leaves, 50-140 mg tissue). DNA was prepared from these six packets as before.

### 2.6.3 DNA from individual transgenic plant lines

Crude DNA samples from individual plant lines were prepared according to Edwards *et al.* (1991) as follows. One leaf was ground for 15 sec with an acrylic pestle in a 1.5-ml microfuge tube. 400 µl extraction buffer containing 200 mM Tris-HCl pH 8.0, 250 mM NaCl, 25 mM EDTA, 0.5 % (w/v) SDS was added and the mixture ground again briefly then vortexed for 5 sec. The homogenate was centrifuged for 1 minute and the supernatant transferred to a clean tube. DNA was precipitated with an equal volume of propan-2-ol, collected by centrifugation and air dried for 15 min. DNA was redissolved in 100 µl of TE (10 mM Tris-HCl, 1 mM EDTA) and 1 µl was used for PCR template.

### 2.6.4 T-DNA and D-enzyme primers

| Oligo name | Location | $t_m$°C / GC% | Primer sequence (5'→3') |
|---|---|---|---|
| 50 | Codon 4 DPE | 57.6°C / 48% | GACTGCCAACAAGTTAAAGAGTCCC |
| 51 | Codon 7 DPE | 59.2°C / 52% | GGATATCACAGTGCAGACGTTTGGG |
| 635-5' | Codon 6 DPE | 53.9°C / 42% | AGTTTGCTCTTCAGTTCACCATTG |
| 31 | Codon 12 DPE | 59.6°C / 48% | CATTCCAGGTGCTCCGATAGATTTTCC |
| 30 | Codon 16 DPE | 57.6°C / 48% | CACAAAGCCGTCCGTACAATGACAA |
| FLB | T-DNA | 58.6°C / 41% | GATGCACTCGAAATCAGCCAATTTT AGAC |
| FRB | T-DNA | 61.4°C / 48% | TCCTTCAATCGTTGCGGTTCTGTCA GTTC |
| TAG5 | T-DNA | 52.7°C / 36% | CTACAAATTGCCTTTTCTTATCGAC |
| TAG3 | T-DNA | 59.2°C / 52% | CTGATACCAGACGTTGCCCGCATAA |

The DPE primers presented in the above table were synthesised by Oswell DNA Service. 635-5' was designed for other purposes but was used in the screening.
Test PCR and expected fragment lengths are illustrated in Figure 2.2.
T-DNA primers were synthesised based on sequence near the ends of T-DNA.

### 2.6.5 PCR amplification and detection of T-DNA/D-enzyme junction sequences

PCR was performed in 25 µl reactions in Hybaid racked Omnitubes (96× 0.3ml tubes) topped with mineral oil. The reaction mixture contained:

PCR Buffer (final concentration: 50 mM Tris-HCl, pH 8.3, 0.5 mg $ml^{-1}$ BSA, 0.5 % (v/v) Ficoll, 1 % (w/v) sucrose, 30 mM KCl, 30 mM $MgCl_2$, 1 mM tartrazine);

200 µM dNTPs;

*Taq* polymerase (made according to Pluthero, 1993);

One T-DNA-specific and one gene-specific primer, each at a final concentration of 400 nM;

50 ng template DNA.

The protocol started with an initial 30 second incubation at 94°C, followed by 35 cycles of PCR (94°C 30 sec, 55°C 30 sec, 72°C 2 min 30 sec). 20 µl of the product were separated on a 1.2 % (w/v) agarose gel 0.5 × 11 × 14 cm with 2 × 20-well combs stacked one above the other 7 cm apart, at 85 V. DNA was transferred onto Hybond $N^+$ (Amersham) nylon membrane by alkali capillary blotting overnight. D-enzyme genomic sequence DNA was amplified with primers 50 and 30 from a D-enzyme genomic clone, gel-purified and labelled with [$^{32}$P]-CTP by the random primer method. The filters were pre-hybridised for 3.5h at 65°C in 5× SSC, 5× Denhardt's, 1 % (w/v) SDS, 1% (w/v) Marvel® freeze-dried skimmed milk powder then hybridised with radio-labelled probe overnight at 65°C with fresh pre-hybridisation mixture. Filters were washed in 2× SSC 0.1% (w/v) SDS, once quickly then twice for 15 min and twice for 10 min in 0.2× SSC 0.1% (w/v) SDS at 65°C. The filters were typically exposed to X-ray film with two amplification screens for 1h.

## 2.7 Construction of D-enzyme expression vector and expression in *E. coli*

### 2.7.1 Construction of over-expression vector

A 1.3 kb partial cDNA clone (corresponding to the region beginning 663 bases upstream of the ATG and including the poly-A tail) was introduced into the translational initiation sequence of expression vector pTrc99A (Pharmacia) at the *Sal*

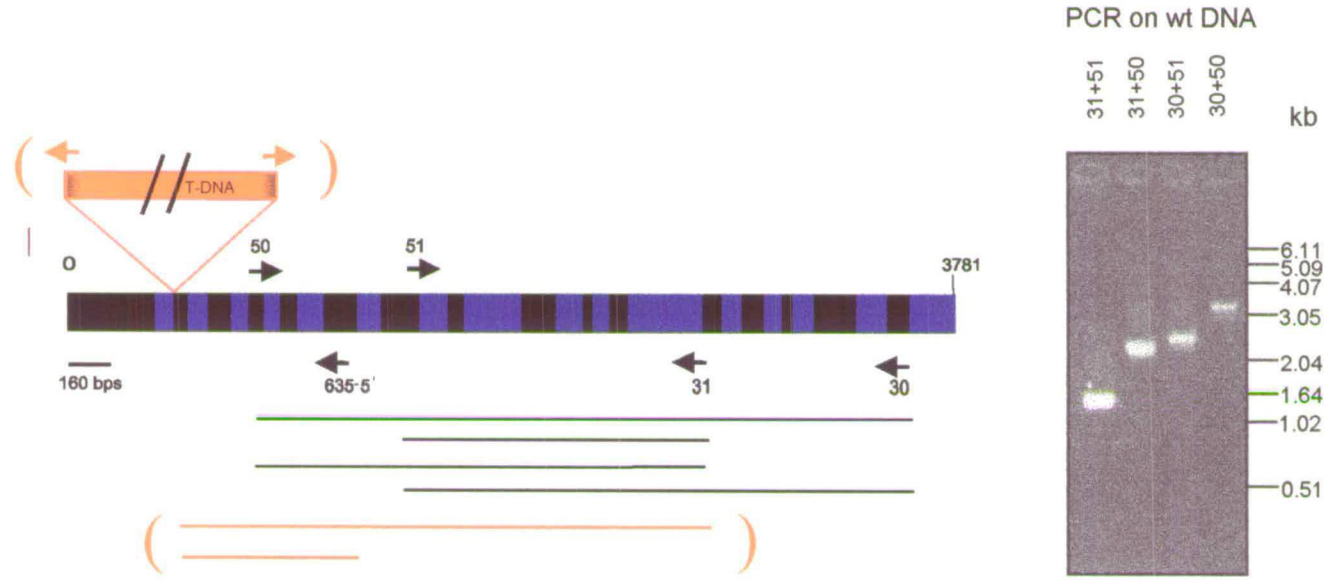



**Figure 2.2 Gene-specific primers: annealing position and fragments amplified in test PCR on wild-type Ws genomic DNA**

Primers designed for the screening of the T-DNA-tagged lines were tested on wild-type Ws genomic (control) DNA. The diagram shows the primer annealing postion within the D-enzyme genomic sequence and fragment lengths obtained (635-5' which was not designed for this purpose was not tested here). T-DNA insertion site and fragment lengths have been included in brackets (described in Chapter 4).

*I* and *Hind III* restriction sites. These sites were derived from the pZL1 vector resulting from *in vitro* excision of the library clone.

The junctions between the multiple cloning site of the vector and the introduced cDNA were sequenced to confirm the identity of the clone prior to expression studies. The expression vector was designated pTrc-DPE.

### 2.7.2 Purification of recombinant *Arabidopsis* D-enzyme from *E. coli*

A single colony of *E. coli* XL1-blue cells carrying the plasmid pTrcDPE was cultured in LB medium containing 100 μg $ml^{-1}$ ampicillin at 37°C, in aerobic conditions, overnight. This overnight culture was used to inoculate 20 volumes fresh medium and grown until $A_{600}$ = 0.4 (~1h) before inducing protein expression by addition of IPTG to a concentration of 1 mM. The culture was then incubated at 37°C for a further 8 h. Cells were harvested by centrifugation and disrupted by sonication in 20 mM Tris-HCl (pH 7.5) containing 5 mM 2-mercaptoethanol at 4°C. This crude extract was centrifuged and the pellet was dissolved in 20 mM Tris-HCl (pH 7.5) and Laemmli buffer (Laemmli, 1970). Proteins contained in the pellet were separated by SDS PAGE on a preparative scale using Protean II xi cell (Biorad) gel apparatus at 35 mA for 6 h. The gel was stained with 0.25% (w/v) Coomassie R, 10% (v/v) acetic acid, 40% (v/v) ethanol and destained in 5% (v/v) acetic acid, 10% (v/v) ethanol, then washed twice with distilled water. The D-enzyme bands were excised from the gel using a sterile scalpel and transferred into a length of semi-permeable membrane (pore size having a 12 kDa exclusion limit) secured at each end with a Medi-clip. Once inside, gel fragments were crushed to facilitate elution. The membrane was placed in an electrophoresis tank containing Tris-Glycine buffer and proteins electro-eluted at 125 V for 5 h. The protein solution was recovered and centrifuged briefly to sediment acrylamide solids. The semi-permeable membrane was rinsed thoroughly with distilled water to remove any remaining solids, then re-clipped and the protein solution returned to it (re-use of the membrane should minimise protein loss since binding sites should have been saturated). The protein solution was dialysed against 5 litres of distilled water at 4°C for 48 h. The protein solution was transferred to a Falcon tube and freeze-dried. The protein was redissolved in sterile water to a concentration of ~1 μg $ml^{-1}$ and maintained at -20°C until required.

### 2.7.3 Immunisation of rabbits and preparation of antisera

Four 1 ml injections each consisting of 250 μg purified protein in water with 50 % (v/v) Freund's complete adjuvent (Sigma) were made to each of two rabbits at 14 day intervals. Blood was collected 14 days after the third and fourth injections and again 28 days after the fourth injection. Injection and blood collection was carried out by staff at the Department of Medical Microbiology, University of Edinburgh. Serum was collected after centrifugation of the clotted whole blood, aliquotted and maintained at -20°C.

## 2.8 Protein isolation, purification and analysis

### 2.8.1 Small scale preparation of total protein from plant tissue

About 0.5g plant tissue was ground in liquid nitrogen and transferred to a 15ml Falcon tube. 1 ml of extraction buffer (20 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol) was added and then the mixture vortexed and centrifuged at 4°C at 1200*g* for 10 min. The supernatant was removed to a fresh microfuge tube and used immediately.

### 2.8.2 Very small scale preparation of total protein from plant tissue

Several leaves were homogenised in 100 μl ice-cold extraction buffer (20 mM Tris-HCl (pH 7.5), 5mM 2-mercaptoethanol) in a microfuge tube with an acrylic mini-pestle. Extracts were vortexed briefly then centrifuged at 14 000*g* for 5 min. The supernatant was removed to a fresh microfuge tube and used immediately for immuno-detection of D-enzyme after SDS-PAGE. In the case of the organ-blot appropriate amounts of tissue and buffer were used, for example in the case of petals, as many petals as possible were ground in 50 μl.

### 2.8.3 Protein assay

The total protein concentration of each extract was determined with respect to a BSA standard curve using the Bio-Rad protein determination kit, which is based on the Bradford assay (Bradford, 1976).

### 2.8.4 SDS-PAGE and measurement of molecular weight

SDS-PAGE was carried out as described in Laemmli (1970), using Tall Mighty Small (Bio-Rad) gel apparatus. Proteins were separated on 1-mm thick, 10 % (w/v) polyacrylamide gels at 140 V. 10 μg protein was loaded per well unless otherwise stated. The relative molecular mass of protein was estimated using unstained (Dalton VII, Sigma) or pre-stained (30-120 000 kDa, Sigma) standards as molecular weight markers.

### 2.8.5 Transfer of proteins to nitrocellulose membrane

Proteins were transferred onto nitrocellulose membrane (0.45μm Protran, Schleicher &Schuell) by electroblotting using a Trans-Blot SD semi-dry transfer cell (Bio-Rad). After SDS-PAGE the gel was incubated in transfer buffer (50 mM Tris, 192 mM glycine, 20 % (v/v) methanol) for 15 min along with a piece of membrane and six pieces of Whatman 3MM paper cut to size. Paper, membrane, gel and paper were arranged as a stack between the plates of the semi-dry blotter and transfer was allowed to proceed for 45 min at 12 V. After transfer, the membrane was rinsed with distilled water and used in immuno-detection.

### 2.8.6 Immuno-detection of D-enzyme on the membrane

All incubations for immuno-detection were carried out at room temperature on a rotary shaker. Membranes were incubated in blocking reagent (TBS buffer with 0.1% (v/v) Tween-20 and 1% (w/v) milk powder) for 1 h, then washed briefly using two changes of TBST (TBS buffer with 0.1% (v/v) Tween-20) and then three times for ~10 min each. Primary antibody incubation was performed with a 1/2000 dilution of serum containing D-enzyme antibody in TBST for 1 h. Membranes were washed twice briefly then once for 15 min and twice for five min in TBST. Secondary antibody incubation was performed with a 1/2000 dilution of anti-rabbit Ig-G-horseradish peroxidase linked whole antibody (Amersham) in TBST for 1h. Unbound secondary antibody was removed with two brief washes and four 5 minute washes in TBST. Detection was carried out with ECL™ (Amersham) according to the manufacturer's handbook. X-ray film was exposed to membranes for 15 and 30 seconds, 1 and 4 min.

### 2.9 Native discontinuous PAGE and activity staining zymograms

Native PAGE was used to separate different isoforms of enzymes of starch degradation. Amylopectin gels, which enable the detection of starch-hydrolysing enzymes, were prepared according to Hill *et al.* (1996). Hydrolysis of amylopectin by different enzymes produces products which stain different colours with iodine (Kakefuda and Duke, 1984): β-amylases create brown bands, debranching enzymes create pale blue bands and endo-amylases create clear colourless bands. Glycogen containing gels were used to separate isoforms of starch phosphorylase as described in Steup (1990). Cytosolic phosphorylase has a high affinity for branched polyglucans such as glycogen and so its migration in glycogen gels is retarded with respect to the plastidic isoform which has a lower affinity for the glycogen. When glycogen-containing native gels are incubated in a developing solution containing G-1-P the phosphorylase isoforms can synthesise amylose which appears as dark blue bands. The separation gel was 7.5% (w/v) acrylamide, 375 mM Tris-HCl (pH 8.8), 0.1% (w/v) ammonium persulphate and 0.005% (v/v) TEMED, and either 0.1% (w/v) potato amylopectin or 0.8 % (w/v) oyster glycogen. Protein was loaded onto the gel with native PAGE loading buffer (final concentration 625 mM Tris-HCl (pH 8.3), 10% (v/v) glycerol, 5% (v/v) 2-mercaptothanol, 0.001% (w/v) BPB). Electrophoresis was carried out using 25 mM Tris-HCl (pH 8.3), 192 mM glycine as running buffer at 4°C at 100 V for about 4 h. Gels were then washed and incubated with the appropriate developing solution at room temperature on an orbital shaker. Amylopectin gels were washed with 50 ml 100 mM Tris-HCl (pH 7.0), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1mM DTT for 15 min then incubated in fresh medium overnight. Starch phosphorylase gels were washed twice in 100 mM sodium succinate pH 6.0, 0.05% (w/v) soluble starch for 10 min and then incubated overnight in this medium additionally containing 20 mM G-1-P. A control gel was included which did not include G-1-P, since native gels can be variable.

### 2.10 Enzyme assays

Enzyme assays were carried out with the help and supervision of Professor Alison Smith and Dr. Samuel Zeeman at John Innes Centre, Norfolk.

Optimum pH and concentrations of all the components of the assays were optimised using crude extracts of wild-type *Arabidopsis* leaves. Leaves, harvested midway

through photoperiod from 6-week-old plants (0.2-0.7g fresh weight) were homogenised using a pestle and mortar at 0-4°C.

### 2.10.1 Endoamylase (α-amylase)

Leaves were extracted in 15 mM calcium acetate, pH 6.0, 1 mM DTT. α-amylase activity was measured by the method of Doehlert and Duke (1983). The 500 µl reaction mixture contained 50 mM MOPS, pH 6.8, 500 units β-amylase (from barley) and 10 mg $ml^{-1}$ starch azure. After incubation at 25°C for 1 h, undigested starch azure was precipitated by adding 2.5 % (w/v) trichloroacetic acid and removed by centrifugation. The absorbance of the supernatant at 595 nm was measured.

### 2.10.2 Pullulanase

Leaves were extracted in 100 mM MOPS, pH 7.2, 2 mM $MgCl_2$, 10 mM DTT, 10 % (v/v) ethanediol, 50 mg $ml^{-1}$ PVPP. The extract was concentrated five-fold using a Microcon-10 microconcetrator (10 kDa molecular mass cut-off). The 200 µl reaction mixture contained 50 mM MOPS, pH 7.2, 10 mM DTT and 20 mg $ml^{-1}$ pullulan. After incubation at 30°C for 2 h, the increase in reducing ends was determined by the dinitrosalicylic acid method of Bernfeld (1955) and compared with maltotriose standards assayed in the same conditions.

### 2.10.3 β-amylase

Leaves were extracted in 50 mM sodium acetate, pH 6.0, 5 mM EDTA, 5 mM DTT. The 500 µl reaction mixture contained 50 mM sodium acetate, pH 5.6, 5 mM EDTA, 5 mM DTT and 10 mg $ml^{-1}$ soluble potato starch. The reaction was stopped by boiling for 10 min. Maltose was hydrolysed by incubation with 2 units maltase (from yeast) for 2 h at 37°C. Glucose concentration was calculated using hexokinase and glucose-6-phosphate dehydrogenase to convert glucose to 6-phosphogluconate and measuring absorbance at 340nm due to the formation of NADH.

### 2.10.4 Starch phosphorylase

Leaves were extracted in 40 mM HEPES-NaOH, pH 7.5, 1 mM EDTA.

The 1 ml reaction mixture contained 20 mM MOPS, pH 7.0, 20 mM $Na_2HPO_4/KH_2PO_4$, 10 mM $MgCl_2$, 3.4 mM NAD, 1 unit phosphoglucomutase (from rabbit muscle), 1 unit glucose-6-phosphate dehydrogenase (from *Leucostoc*

*mesenteroides*), 4 mM glucose-1, 6-bisphosphate and 2.5 mg $ml^{-1}$ amylopectin (Steup, 1990).

### 2.10.5 Maltase (α-glucosidase) and D-enzyme (4-α-glucanotransferase)

Leaves were extracted in 20 mM MES, pH 6.2. The 500 µl reaction mixture contained 50 mM sodium acetate, pH 5.2 and 90 mM maltose for maltase (Kruger and ap Rees, 1983) or 50 mM MOPS, pH 6.8, 60 mM maltotriose for D-enzyme (Takaha *et al.*, 1993). Assays were stopped by boiling and glucose was determined as described for β-amylase.

### 2.10.6 Soluble starch synthase

Leaves were extracted in 100 mM MOPS, pH 7.2, 1 mM EDTA, 1 mM DTT, 10% (v/v) ethanediol, 50 mg $ml^{-1}$ PVPP. The resin method of Jenner *et al.* (1994) was used, except that the pH was 8.3 and the amylopectin concentration was 18 mg $ml^{-1}$.

### 2.10.7 Starch-branching enzyme

Leaves were extracted as described for soluble starch synthase and the phosphorylase stimulation assay, as described by Smith (1988) was used.

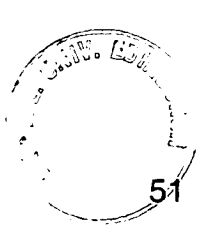
## 2.11 Preparation of tissue and starch for electron microscopy

### 2.11.1 Preparation of leaf starch granules

*Arabidopsis* leaves from 6 week old plants (20 g) were harvested at the beginning and at the end of their 10 h photoperiod, washed and homogenised with a hand-held blender in five volumes of 100 mM MOPS, pH 7.2, 5 mM EDTA, 10% (v/v) ethanediol at 4°C. The homogenate was filtered through two layers of Miracloth and a 20 µM nylon mesh, centrifuged (10 min, 4°C, 3000 *g*) and the pellet resuspended in 30 ml of the same medium plus 0.5 % (w/v) SDS by vortexing. The starch was pelleted by centrifugation as before, washed twice more with SDS-containing medium and then six times with deionised water. Starch was resuspended in acetone and allowed to dry on the surface of 12 mm aluminium EM stubs. Starch was gold sputter-coated and analysed under a Cambridge Stereoscan 250 scanning electron microscope. Images were captured digitally using an I-SCAN interface (ISS Ltd.).

# Chapter 3

# Isolation and Characterisation of an *Arabidopsis* D-enzyme Gene



### 3.1 Introduction and aims

D-enzyme acts on starch *in vitro*. *In vivo*, it appears to be present where starch is accumulated. D-enzyme mRNA expression in the leaves of potato is induced by light and sugars and follows a similar pattern to those of plastidic starch phosphorylase and granule bound starch synthase (Takaha, 1996). Much evidence suggests that it has a role in starch metabolism. However, in transgenic potato plants with sense and antisense copies of the potato D-enzyme gene which had only 1% wild-type D-enzyme activity, starch content of tubers was not appreciably altered in amount, proportion of amylose, molecular weight of debranched amylopectin, or branch chain length. D-enzyme activity is normally high (the ratio of D-enzyme activity to total amylolytic activity is 1.5 : 1 in *Arabidopsis* chloroplasts and 1.1 : 1 in pea chloroplasts), therefore it is possible that 1% of activity could be sufficient for normal starch metabolism. In potato, reduction of D-enzyme resulted in slow plant growth, and delayed sprouting of tubers. Although these results are consistent with a role for D-enzyme in starch metabolism, that role remains obscure.

The aim of this work was to investigate the role of D-enzyme further by obtaining a null mutant lacking all D-enzyme activity. The aim of the work described in this chapter was to obtain experimental materials necessary for the isolation of a D-enzyme null mutant plant and for analysis of D-enzyme protein expression in the mutant and wild-type plants. It was necessary to obtain cDNA and genomic clones for D-enzyme in *Arabidopsis* mainly for sequence information allowing the design of primers for the screening of collections of T-DNA insertion mutants, described in Chapter 4. The sequence also enabled molecular biological analysis. Although a number of bacterial amylomaltase sequences had been isolated at the start of this study, the only higher plant D-enzyme sequence isolated was that of potato. Potato D-enzyme shows significant homology with amylomaltases from *Clostridium butyricum* (40.2% amino acid sequence identity) and from *Streptococcus pneumoniae* (36.6%) but less homology with those from *Escherichia coli* (11.6%) and *Haemophilus influenzae* (10.2%) (Takaha, 1996).

Antibodies raised against D-enzyme were required for several purposes. Firstly, at the time of screening, the 5'end of the D-enzyme gene sequence was unknown. The normal method of distinguishing plants homozygous for the D-enzyme mutation from those that are heterozygous required a primer in this unknown region, upstream of the T-DNA insert. Therefore homozygous mutants were identified from the progeny of the D-enzyme mutant plant by western analysis. Secondly, the expression of D-enzyme in wild-type *Arabidopsis* had not been fully characterised and had been investigated in leaves alone. 80% of D-enzyme activity in *Arabidopsis* leaves is reported as chloroplastic (Lin *et al.,* 1988). Two forms of D-enzyme have been purified from *Arabidopsis* leaves, one with four times the specific activity of the other (Lin and Preiss, 1988). It was of interest to determine if a second immuno-reactive D-enzyme was present in the D-enzyme mutant isolated, and to localise D-enzyme expression within the plant and within the cell.

### 3.2 Isolation of D-enzyme partial cDNA clone

#### 3.2.1 Isolation of cDNA clones for D-enzyme

A full-length potato D-enzyme cDNA sequence (gift of Takeshi Takaha, Ezaki Glico Co., Japan) was used as a hybridisation probe to screen a cDNA library. The λ PRL2 cDNA library (obtained from the Nottingham Stock Centre) contained cDNA synthesised from poly $A^+$ RNA isolated from all developmental stages of wild-type *Arabidopsis* thaliana (L.) cv. Columbia and was constructed in bacteriophage lambda vector, λZiplox™. Five positively hybridising plaques were isolated from 100 000 recombinant plaques. All five clones had cDNA inserts of about 1.3 kb and encoded D-enzyme as shown by partial nucleotide sequencing. No differences in nucleotide sequence were detected in the 5' ends of each cDNA (one clone was 20 bases shorter than the others were) and all contained poly A tails. One clone, pADE-924 was selected for detailed analysis.

#### 3.2.2 Isolation of further cDNA clones for D-enzyme

*Arabidopsis* D-enzyme sequence was obtained by PCR from the pADE-924 clone and used as a hybridisation probe to screen another cDNA library. The Weigel cDNA library (obtained from the Nottingham Stock Centre) contained cDNA synthesised from poly $A^+$ RNA isolated from whole flowers of *Arabidopsis thaliana* (L.) cv. Landsberg *erecta* and was constructed in bacteriophage vector λZAP™. Five positively hybridising plaques were isolated from 700 000 recombinant plaques. The longest clone was also about 1.3 kb.

#### 3.2.3 Sequence analysis of partial D-enzyme cDNA clone

The sequence of pADE-924 was determined completely. It consisted of an open reading frame of 1071 nucleotides, a 3' untranslated sequence of 169 nucleotides and a poly A tail.

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Key: blue sequence denotes intron or non-coding sequence
a, b of c denotes frame of translation of exons in row

**Figure 3.1**
**The complete genomic sequence and the open reading frame encoding the D-enzyme 1 protein**

### 3.3 Isolation and analysis of D-enzyme genomic clone

#### 3.3.1 Isolation of genomic clones for D-enzyme

*Arabidopsis* partial D-enzyme sequence was obtained by PCR from the pADE-924 clone and used as a hybridisation probe to screen a genomic library. The Meyerowitz library (gift of Justin Goodrich, University of Edinburgh) contained genomic DNA from ecotype Columbia and was constructed in bacteriophage lambda vector λGEM®11. Nine positively hybridising plaques were isolated from 100 000 recombinant plaques. The insert DNAs of these nine were analysed by digestion with restriction enzymes and Southern blotting using the cDNA as a probe. The sequence of the genomic clone was determined by ABI Prism cycle sequencing.

#### 3.3.2 Analysis of the DPE genomic sequence

Figure 3.1 shows the complete genomic sequence and the open reading frame encoding the D-enzyme 1 protein. The D-enzyme 1 gene was sequenced during the *Arabidopsis* sequencing project and its complete sequence was obtained using a BLAST search (Altschul *et al.*, 1997) with the cDNA sequence as a query. The Genbank accession number of the genomic sequence is AB019236. This database sequence was used together with that obtained from the genomic clone. The gene has been mapped to chromosome 5-86.5 (Jychian Chen, personal communication) and RFLP analysis indicates that the gene is single copy. The genomic sequence consists of 16 exons and 16 introns.

#### 3.3.3 Analysis of the promoter region of the D-enzyme gene

The putative promoter region of the D-enzyme gene was analysed using the Plant cis-acting regulatory DNA elements (PLACE) database (Higo e*t al.*, 1999) at http://www.dna.affrc.go.jp/htdocs/PLACE on the World Wide Web. Elements found are indicated in Figure 3.2 and described in the accompanying legend.

Sequence elements in the promoter region suggest that D-enzyme is plastid localised, related to the α-amylase genes and probably light regulated.

### 3.4 Isolation of 5'end of cDNA by 5'RACE

The 5' sequence of the D-enzyme cDNA was isolated by 5'RACE using 5'RACE System for Rapid Amplification of cDNA ends (Gibco BRL Life Technologies), Incorporated. cDNA was synthesised from poly $A^+$ RNA isolated from inflorescence tips of Arabidopsis ecotype Landsberg erecta. This work was carried out with Justin Goodrich (University of Edinburgh). The sequence of the 5' end-containing clone consisted of 48 nucleotides followed by an open reading frame of 450 nucleotides.

### 3.5 PCR on cDNA to obtain full length cDNA sequence in Ws ecotype

The T-DNA tagged plant lines were prepared from *Arabidopsis thaliana* (L.) cv. Wasserilewskija (Ws). PCR was used to amplify complete D-enzyme sequence from Ws cDNA template. Primers were designed using the combined sequence obtained from the 5'end cDNA clone, the genomic clone and the partial cDNA clone pADE-924. cDNA was synthesised from total RNA isolated from wild-type *Arabidopsis* ecotype Ws leaf tissue.

### 3.6 Sequence analysis

The cDNA sequence of D-enzyme 1 was determined from Ws PCR product and provides part of Figure 3.1.

#### 3.6.1 Homology with potato D-enzyme

The *Arabidopsis* D-enzyme 1 peptide sequence was aligned with potato, the only other available plant D-enzyme sequence. The alignment is shown in Figure 3.3.

#### 3.6.2 Homology with a second *Arabidopsis* putative D-enzyme sequence

A second putative D-enzyme gene (notated D-enzyme 2) was sequenced during the *Arabidopsis* sequencing project and its complete sequence was obtained using a BLAST search (Altschul *et al.*, 1997) using the D-enzyme 1 cDNA sequence as a query. The Genbank accession number of the sequence is ATAC002409. The sequence is located on chromosome 2. The amino acid sequence deduced from the sequence of *DPE2* was aligned with the *DPE1* peptide and this alignment is shown in Figure 3.4.

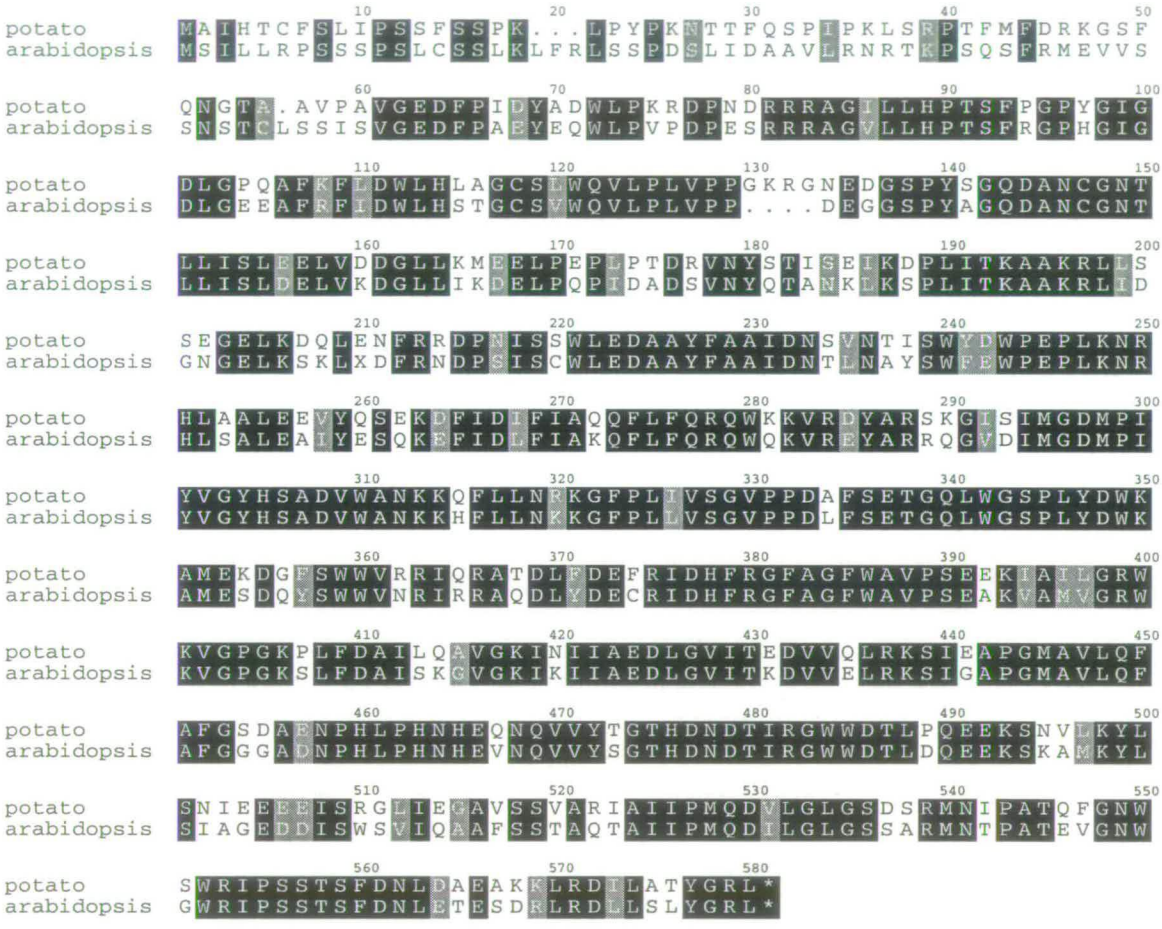
**Figure 3.2 Sequence of the putative promoter region of the D-enzyme 1 gene and transcription elements identified within it.**

- Polyadenylation signals
  AATAAA
  AATAAT
  AATTAAA
  also found in rice, pea and maize α-amylase (Heidecker and Messing, 1986; Joshi, 1987; O'Neill *et al.*, 1990).
- CAAT / CCAAT boxes
- AMYBOX 'amylase box'; amylase element;
  TATCCAT
  conserved sequence found in 5' upstream region of α-amylase genes of rice, wheat, barley (Hwang *et al.*, 1990, 1998).
- CGACGOSAMY3
  CGACG element
  element found in the GC rich regions of the rice Amy3D α-amylase gene; may function as a coupling element for the G box element; required during sugar starvation (Hwang *et al.*, 1998).
- S1F-BOX
  ATGGTA
  negative cis-element conserved in plastid-related genes (Zhou *et al.*, 1992).
- BOX1PVCHS15
  TAAAAGTTAAAAAC
  involved in organ-specific expression in plant development; resembles the binding site for the GT-1 factor in light responsive elements (Villain *et al.*, 1996) functions as a transcriptional silencer in electroporated protoplasts derived from undifferentiated suspension-cultured soybean cells (Lawton *et al.*, 1991).

Other sequences (unmarked) include:

- GATAMOTIFCAMV
  GATA (21 copies)
  motif in cauliflower mosaic virus 35S promoter related to binding with ASF-2 (Lam and Chua, 1989); may be involved with light regulation of transcription (Gilmartin *et al.*, 1990).
- GT1 CONSENSUS
  GRWAAW (13 copies)
  GT-1 binding site found in many light-regulated genes (Villain *et al.*, 1996; Terzaghi and Cashmore, 1995).
- IBOXCORE
  GATAA (7 copies)
  conserved sequence in light regulated genes (Giuliano *et al.*, 1988).


**Figure 3.2 DNA sequence of the promotor region of the D-enzyme gene**




## Figure 3.3 Alignment of the *Arabidopsis thaliana* and *Solanum tuberosum* D-enzyme sequence

The deduced amino-acid sequence of *Arabidopsis* D-enzyme was aligned with that from potato (Takaha *et al.*,1993). Box-and-shading was performed using JavaShade (Southern and Lewis, 1998) at http://industry.ebi.ac.uk/JavaShade on the World Wide Web. Black boxes indicate identical amino acids and grey boxes denote amino acids with similar chemical properties according to Zvebil *et al.* (1987).

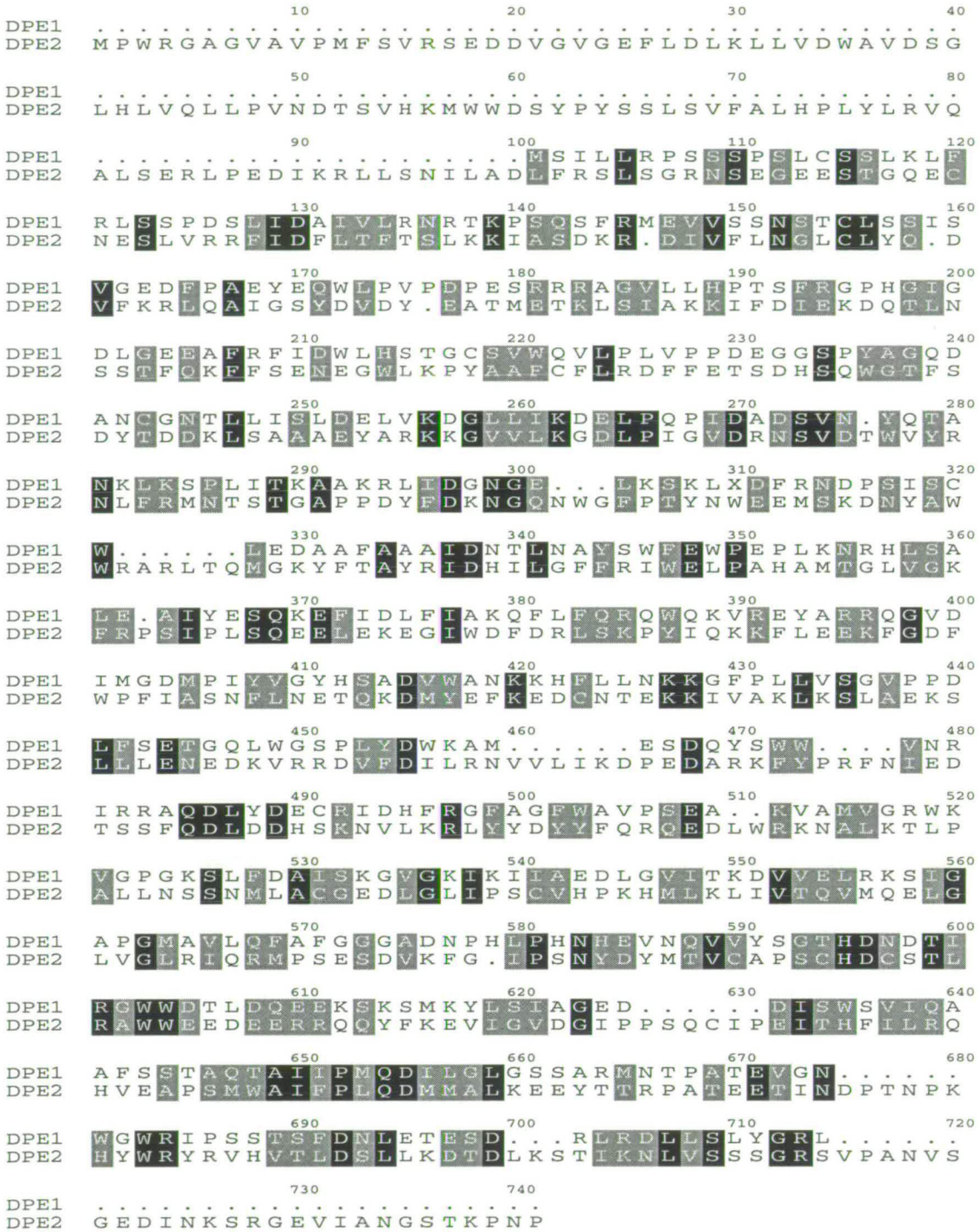



**Figure 3.4 Alignment of the *Arabidopsis thaliana DPE 1* with putative D-enzyme sequence *DPE 2***

The deduced amino-acid sequence of *Arabidopsis* D-enzyme *DPE 1* was aligned with that deduced from genomic sequence ATAC002409 obtained during sequencing of *Arabidopsis* chromosome 2. Box-and-shading was performed using JavaShade (Southern and Lewis, 1998) at http://industry.ebi.ac.uk/JavaShade on the World Wide Web. Black boxes indicate identical amino acids and grey boxes denote amino acids with similar chemical properties according to Zvebil *et al.* (1987).

### 3.7 Features of the D-enzyme polypeptide

#### 3.7.1 N-terminal extension sequence

In *Arabidopsis* leaves at least 80% D-enzyme activity was found in the chloroplast fraction (Lin *et al.*, 1988). The mature polypeptide detected in crude extracts of *Arabidopsis* by western analysis has a molecular mass of about 60 000 Daltons. The enzyme is apparently made as a higher molecular weight precursor of 64,641 Daltons (calculated from the amino acid content).

Comparison of the *Arabidopsis* and potato D-enzyme sequences indicates that there was poor homology between the first 180 bases (encoding the first 60 amino acids, Figure 3.3) The potato D-enzyme sequence encodes a 52 residue transit peptide and transit peptide sequences are often poorly conserved. Homology in the alignment between the two begins at the amino-acid sequence 'VGEDFP', 60 amino acids after the ATG in the *Arabidopsis* sequence. This 60 amino-acid sequence was submitted to ChloroP V1.0 Chloroplast Transit Peptide Prediction Centre for Biological Sequence Analysis at http://www.cbs.dtu.dk/services/ChloroP/ on the world wide web (Emanuelsson *et al.*, 1999) to determine whether it is likely to function as a transit peptide. The program predicted that the sequence was likely to function as a transit peptide and predicted a cleavage site between residues 45 and 46. Cleavage of the transit peptide would account for the observed molecular weight of the mature polypeptide of 527 residues ($M_r$ 59, 500).

#### 3.7.2 Comparison of D-enzyme with starch and glycogen metabolic enzymes

Enzymes which catalyse hydrolytic or transglycosidic reactions on starch (including amylases, cyclodextrin transferases, debranching enzymes and branching enzymes) contain four regions of conserved sequence, and have been named the α-amylase family of enzymes (Takata *et al.*, 1992). X-ray crystallographic structural analysis and protein engineering studies demonstrated that the conserved amino acids in these regions play important roles in the catalytic mechanism of the enzymes (Matsuura, 1995). The *Arabidopsis* D-enzyme sequence, like potato D-enzyme and bacterial amylomaltases (Takaha, 1996) contains three of these four conserved regions.

These regions are: $^{369}$ ECRIDHFRG, $^{420}$ EDLG, and $^{466}$ YSGTHD

It would appear that D-enzymes do not contain the first of the conserved regions and so the mechanism of catalysis may vary slightly from the α-amylase family.

### 3.8 Expression of recombinant D-enzyme cDNA in *E.coli* and antibody production

The 1.3 kb partial D-enzyme cDNA clone obtained from the λPRL2 cDNA library was used to prepare antibodies against *Arabidopsis* D-enzyme to be used in later analysis of transgenic plants and of gene expression. A partial cDNA sequence was incorporated into *E.coli* expression vector pTrc99A and the resultant vector pTrcDPE was introduced into *E.coli* XL1-blue cells. Following induction of gene expression with IPTG, a maximum yield of the over-expressed truncated protein (40.2 kDa) was obtained from cultures grown for 7-8 hours (see Figure 3.5- expression time course gel). The protein accumulated in the cells as insoluble inclusion bodies and was purified from the insoluble (pellet) fraction of sonicated culture by preparative SDS PAGE, electro-elution and dialysis as described in section 2.9. Figure 3.6 shows the overexpressed protein in induced cells, in the pellet fraction, and after purification. The purified protein was injected into a rabbit four times at 14 day intervals and antisera were prepared 14 days after the third and fourth injections and 28 days after the fourth injection. The specificity of the antisera was checked by western blot analysis against *Arabidopsis* crude extracts, where a 59 kDa polypeptide (the size predicted for mature D-enzyme) was specifically detected.

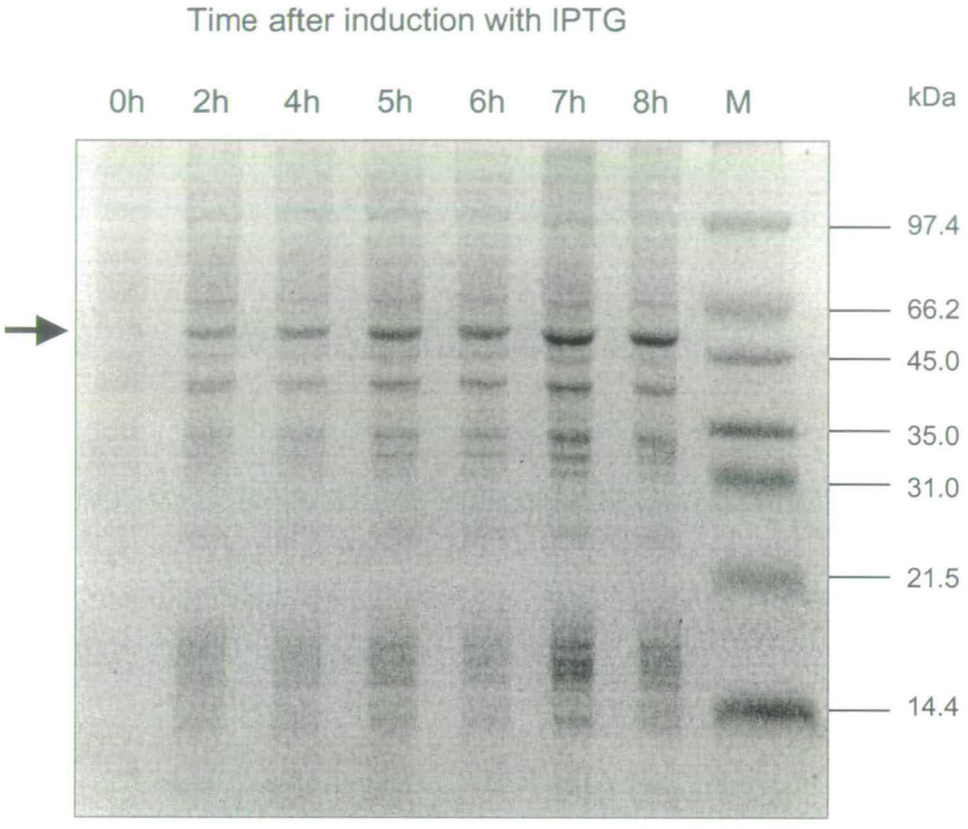



**Figure 3.5 Time course of expression of partial D-enzyme cDNA in *E. coli***

*E. coli* XL1-blue cells carrying the plasmid pTrcDPE were cultured in LB medium at 37°C until mid-log phase then IPTG (1 mM) was added to induce protein expression and the culture grown at 37°C for a further 8 h. Samples were removed 2,4,5,6,7 and 8 h after induction. Cells were harvested by centrifugation, washed and resuspended in 20 mM Tris-HCl (pH 7.5). Equal volumes of whole cell extract were separated by SDS PAGE on a 10% acrylamide gel at 20 mA, stained with Coomassie R and destained.

The arrow indicates the overexpressed truncated D-enzyme increasing to a maximum at 7-8 h after induction.

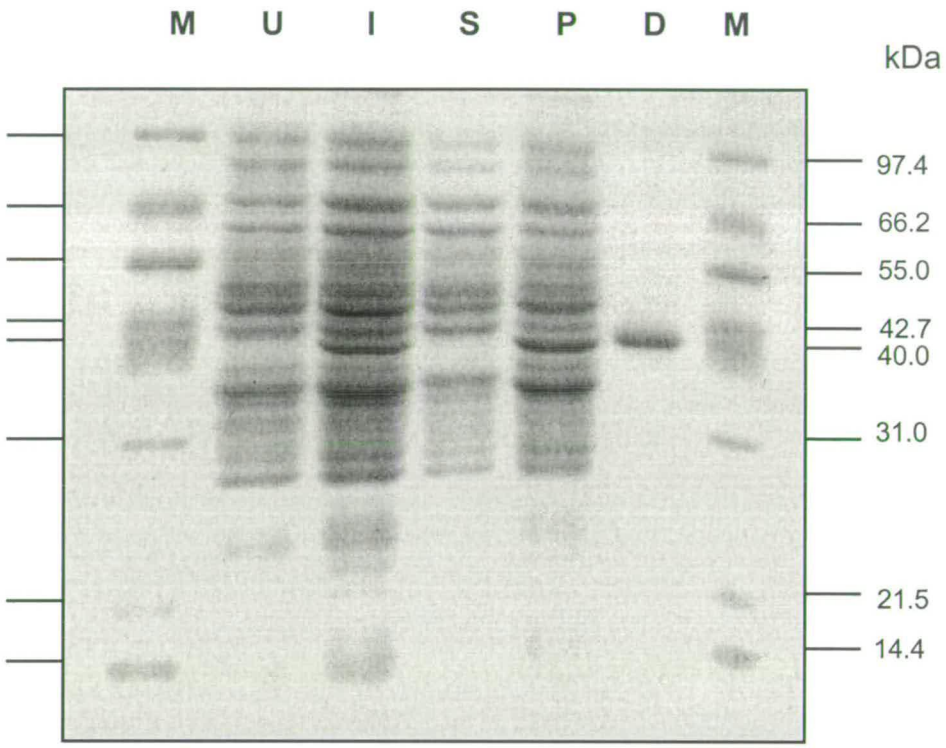



**Figure 3.6 Expression of partial D-enzyme cDNA in *E. coli***

*E. coli* XL1-blue cells carrying the plasmid pTrcDPE were cultured in LB medium at 37°C until mid-log phase (**U**-uninduced), then IPTG (1 mM) was added to induce protein expression and the culture grown for a further 8 h at 37°C (**I**-induced). Cells were harvested by centrifugation and disrupted by sonication in 20 mM Tris-HCl pH 7.5, then centrifuged to separate supernatant (**S**) and pellet (**P**). Pellet was separated on preparative SDS PAGE gel and stained with Coomassie R. The band corresponding to D-enzyme protein was excised, crushed and placed in dialysis tubing. Protein was electro-eluted at 125 V for 5 h in Tris-Glycine buffer, dialysed against $H_2O$ overnight and freeze-dried. Protein was reconstituted in water to 1 mg $ml^{-1}$ (**D**) Samples corresponding to equivalent cell mass were separated by SDS PAGE on a 12% acrylamide gel stained with Coomassie R and destained.

### 3.9 Conclusion

A cDNA clone encoding an *Arabidopsis* D-enzyme was isolated from a library using oligonucleotide probes derived from the full-length potato D-enzyme cDNA sequence. The identity of the cDNA sequence as encoding D-enzyme was confirmed by the high degree of amino-acid identity with the potato D-enzyme as deduced from the cDNA sequences. The sequence had some homology with a second *Arabidopsis* putative glucanotransferase sequence. The amino acid sequence deduced from the cDNA indicates the presence of an amino-terminal plastid transit peptide of 45 amino acid residues and a mature polypeptide of 527 residues. Recombinant D-enzyme protein was purified from *E. coli* cells and used to prepare antibodies against D-enzyme. Western blot analysis of crude *Arabidopsis* extracts specifically detected a 59 kDa polypeptide (the size predicted for mature D-enzyme). Genomic clones for D-enzyme were isolated and sequenced. The promoter region of the gene was analysed and the full gene sequence consisting of 16 exons and 16 introns was determined. The genomic and cDNA clones and the antibody described in this chapter enabled the work described in the following chapters to be undertaken.

# Chapter 4

# Isolation of a D-enzyme Mutant *Arabidopsis* Plant by PCR Screen

### 4.1 Introduction

Gene tagging is a technique by which mobile or introduced DNA, with a known sequence, inserts into a gene. The presence of foreign DNA mutates the gene and normal transcription is disrupted: the known sequence also facilitates the isolation of flanking DNA.

Transposons were the first insertional mutagen to be used for this purpose since the phenomenon occurs naturally in a variety of organisms such as bacteria, *Caenorhabditis elegans, Drosophila*, *Antirrhinum majus* and *Zea mays* (reviewed by Berg and Howe, 1989). Transposons are short, mobile genetic elements that can move from one site to another in the genome producing a variety of mutations that have been invaluable to researchers since before the discovery of DNA itself.

In *Arabidopsis*, which harbours no endogenous active transposons, the T-DNA (transferred DNA) of *Agrobacterium tumefaciens* has been used as an insertional mutagen. T-DNA is a segment of the tumour-inducing (Ti) plasmid, which integrates into the genome of a plant cell during infection causing it to proliferate and synthesise nutrients for the *Agrobacterium*. T-DNA has been engineered to remove the tumour-promoting and biosynthetic genes and instead these are replaced with an antibiotic resistance marker to allow the selection of transformed plants or tissues.

Over 14 000 independent T-DNA insertion mutants have been produced using whole-plant transformation as developed by Feldmann and Marks (1987). By this method the germinating seed is infected/transformed, circumventing the tissue culture process which can induce a high frequency of somaclonal variants (Feldmann and Marks, 1986). A further 25 000 transformants have been produced by more recent (and generally more efficient) methods which involve direct application of *Agrobacterium* to the plant, again avoiding tissue culture. Bechtold *et al.* (1993) used vacuum infiltration to transform adult plants; other methods involve severing apical or inflorescence shoots at their bases and inoculating the severed sites with *Agrobacterium* – newly formed shoots at the wound site produced transformed progenies (Katavic *et al.*, 1994; Chang *et al.*, 1994). Genetic characterisation of a

subset of the Feldmann transformants indicates that they contained an average of 1.5 inserts each as assayed by kanamycin resistance. Molecular analysis shows that the inserts are predominantly concatamers of T-DNA arranged as direct and inverted repeats (Feldmann 1991).

Since mutations derived from insertional mutagenesis are tagged and allow rapid isolation of mutant genes, initially this method was used to isolate genes after phenotypic mutant screens. Transposons were first used to isolate bacterial genes (Kleckner, 1981) and have been used to isolate a variety of other genes since. Berg and Howe (1989) review their use for this purpose. The first mutant isolated using T-DNA insertion mutagenesis was *dwarf1* (Feldmann *et al.*, 1989) and many more have followed. For many genes, however, phenotypic screens are not available. For others, as is the case of starch metabolism, many different mutants may give the same broad phenotypes.

Later, a reverse genetics approach was developed enabling the isolation of mutations in genes independently of mutant phenotype but it requires knowledge of the target gene sequence. Polymerase chain reaction (PCR) was used to screen for P-element mutations in sequenced genes of *Drosophila* (Ballinger and Benzer, 1989). McKinney *et al.* (1995) and Krysan *et al.* (1996) developed methods for sequence-based identification of T-DNA insertion mutations in *Arabidopsis*, which use PCR to amplify the junctions between a T-DNA insert and the gene of interest from pools of mutant plant lines. PCR-based reverse genetics using T-DNA insertion mutagenesis in *Arabidopsis* is reviewed in Azpiroz-Leehan and Feldmann (1997). The general PCR strategy is shown in Figure 4.1. A primer homologous to the end of the inserted T-DNA and one primer within the target gene are used to amplify sequences at the junction of the insertion. Multiple combinations of T-DNA and target gene primers can be used in separate reactions to cover all possible combinations of T-DNA insert orientation and position within the gene. Mutant plants are arranged into groups or pools. These pools are grouped into larger 'superpools', which in turn, are arranged into larger 'megapools' and thus a hierarchy of pools is produced. DNA is prepared from the pools and PCR is carried out using

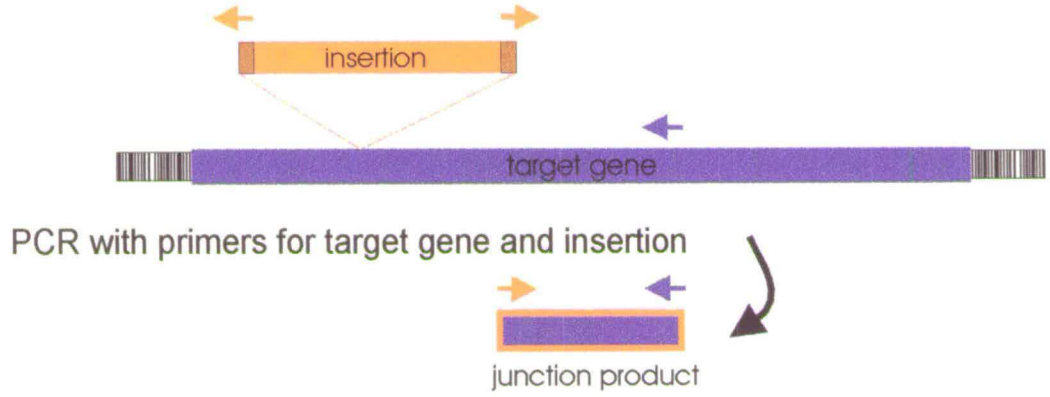



**Figure 4.1 Method of isolation of T-DNA insertions proximal to known gene sequences**

The general method relies on the power of PCR to amplify the junction between T-DNA insert and known target gene sequence from large pools of randomly inserted T-DNA elements.

DNA template from each level of the hierarchy. The PCR products are transferred to nylon membrane and probed with radiolabelled gene of interest, which should hybridise with bands containing sequence from that gene. Once a 'megapool' is identified as positive the next PCR experiment is carried out using DNA from its component superpools. Eventually DNA from individual plants is examined.

This approach was used to screen for a D-enzyme insertion mutant from collections of T-DNA insertion mutants made by Feldmann (1991) (referred to from hereon as Feldmann/Dupont lines) and Bechtold *et al.* (1993) (referred to as Versailles lines).

### 4.2 Isolation of the D-enzyme mutant plant

A diagrammatic representation of the screening progression described next is shown in Figure 4.2.

19 000 T-DNA-containing plant lines were screened initially as 5 'megapools' each containing DNA from 4-5000 plant lines (designated L1-L5). Each 'megapool' DNA template was subjected to PCR amplification with 10 primer pairs in 10 different reactions (in each case one T-DNA primer – denoted TAG5 or TAG 3 (Versailles lines), FLB (Feldmann Left Border) or FRB (Feldmann Right Border) (Feldmann/Dupont lines), and one gene-specific primer of the nested series 50, 51, 635-5', 31 and 30). These primers are detailed in Section 2.6.4, and annealing positions of these primers within the D-enzyme wild-type genomic sequence is shown in Figure 2.2. Products were blotted onto nylon filters, and the filters probed with a PCR derived D-enzyme partial genomic sequence. Two positive signals in 'Megapool' L4 with FLB and gene specific primers 635-5' and 31 were easily detected on short exposures of the autoradiogram (Figure 4.3). These corresponded to PCR products of <1kb and >3 kb respectively. Also in L4 a clear positive signal was identified with FRB and 30 although not corroborating those given with the FLB primer, and it was later shown to be non-specific by repeat PCR (data not shown). Other non-specific products were also observed (for example with FRB and 50 in all Megapools).

'Megapool' L4 was screened again with FLB plus 635-5' or 31 along with its component 'Superpools': FH, FI, D1, D2, D3 and D4. The <1kb and >3kb products were confirmed in L4 and also in 'Superpool' D2 (Figure 4.4). The pools comprising D2 were screened and the <1kb product confirmed in pool 2400 (Figure 4.5). These products were resolved on an agarose gel and the bands excised from the gel, purified and sequenced using the FLB primer. Sequencing confirmed that the insertion was in the D-enzyme gene.

To isolate the individual plant from the pool of 100 lines it was necessary to obtain the corresponding seed and prepare further sub-pools to screen. 12 sub-pools each containing DNA from 60 plants were screened and 6 of these gave a positive result (Figure 4.6). Two of these six were chosen to divide into smaller groups to screen (Figure 4.7) until finally PCR was carried out on DNA from individual plants, identifying two sibling mutant plants, 4.3H (Figure 4.8) and 1.1J (data not shown).

**Figure 4.2 Diagram showing the screening through hierarchical pools of DNA leading to isolation of the D-enzyme insertion mutant**

This figure is intended to be used in conjunction with the experimental results shown in Figures 4.3-4.9. PCR was carried out with pairs of primers (one T-DNA and one gene specific) on pools of DNA of decreasing magnitude. On identification of the smallest pool that gave positively hybridising PCR products consistent with an insertion in the D-enzyme gene, it was then necessary to isolate one plant from seed (of which one in one hundred should contain the desired tagged gene). Plants were grown and one leaf from each plant removed. These leaves were subpooled: sixty plants per tray contributed sixty leaves to one DNA preparation that was used for one PCR. Six out of the twelve PCR reactions produced the expected 0.6 kb product (vertical green arrow). Since the plants responsible for this product were siblings and expected to be identical only two of the six trays were further subpooled (bold vertical green arrow): twenty plants contributed twenty leaves to one DNA preparation. Six more PCR reactions indicated which group of twenty plants contained the mutant. Finally, one leaf from each of the twenty plants was used to provide template DNA for twenty PCR reactions, which identified the individual plant carrying an insertion in the D-enzyme gene.

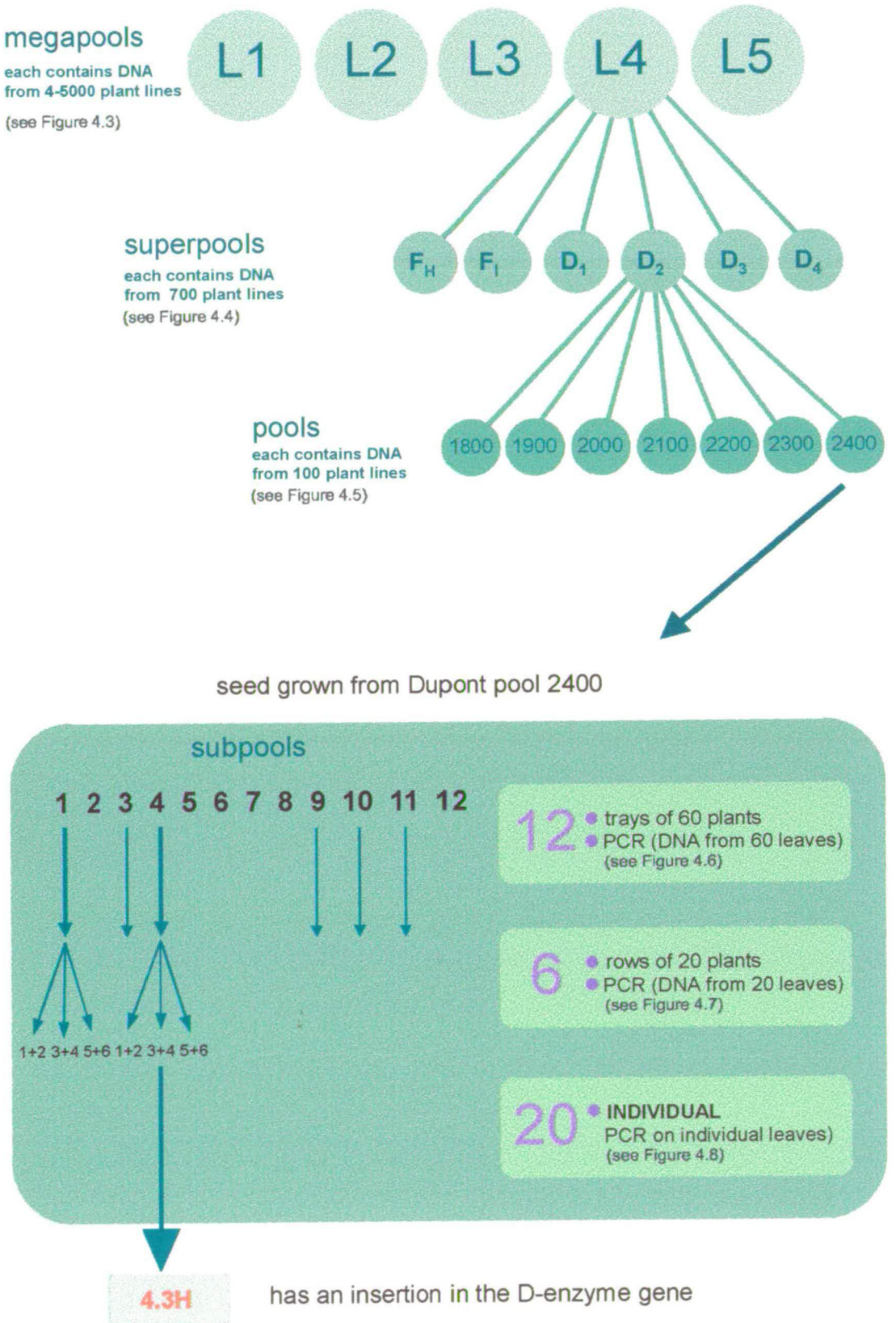



**Figure 4.2 Diagram showing the screening through hierarchical pools of DNA leading to isolation of the D-enzyme insertion mutant**

**Figure 4.3 First round (Megapool) screening**

Southern blot of PCR products probed with D-enzyme genomic sequence.

DNA from 19 000 T-DNA-containing plant lines was screened as 5 'megapools' (L1-L5). For each megapool template 10 different PCR reactions were carried out each containing one T-DNA primer homologous with either the left or right border and one gene specific primer of a nested series where 50 is most 5' and 30 is most 3' within the gene (see Figure 2.2). The Versailles and Feldmann/Dupont lines are from different sources and so different T-DNA primers are used in each case. Arrows indicate positively hybridising products; bold arrows nested products consistent with an insertion in the D-enzyme gene.

Positive controls were performed on wild-type Ws DNA.

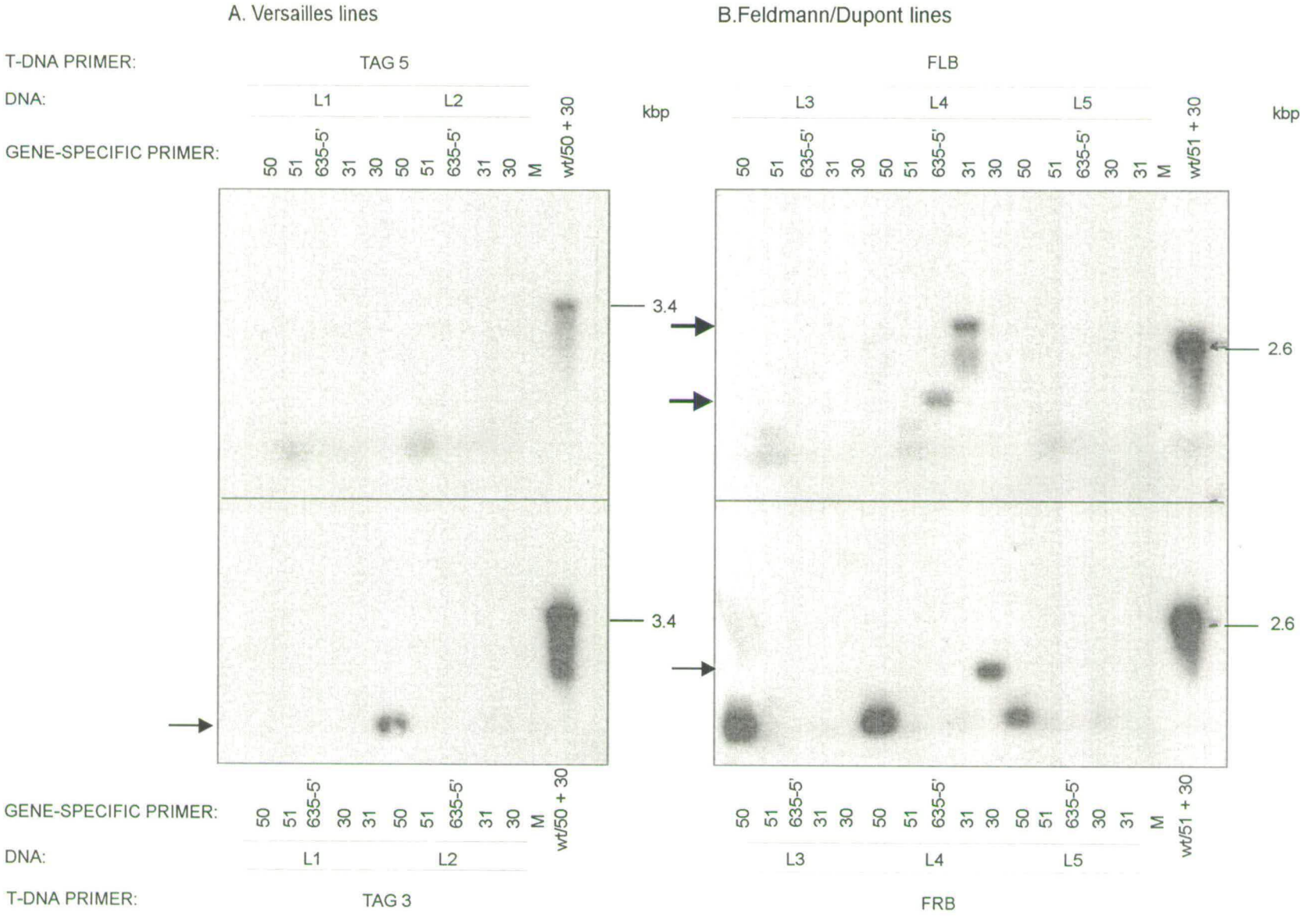

A. Versailles lines
B.Feldmann/Dupont lines
T-DNA PRIMER:
TAG 5
FLB
DNA:
L1
L2
L3
L4
L5
GENE-SPECIFIC PRIMER:
50
51
635-5'
31
30
M
wt/50 + 30
wt/51 + 30
kbp
3.4
2.6
TAG 3
FRB

**Figure 4.4 Second round (Superpool) screening**

Southern blot of PCR products probed with D-enzyme genomic sequence.

Megapools and primer pairs giving positively hybridising products in first round screen were subject to repeat PCR (first lane in each block). The Superpools comprising these Megapools were also screened.

Bold arrows show the nested 0.6 kb and ~3 kb D-enzyme/T-DNA products from the *dpe1*-1 mutant in Megapool L4 and in Superpool D2 (Dupont 2). Other positively hybridising PCR products from the first round screen were not confirmed by repeat PCR.

Positive controls were performed on wild-type Ws DNA.

Negative controls were performed on TE (no template).

**Figure 4.5 Third round (Pool) screening**

Southern blot of PCR products probed with D-enzyme genomic sequence.

Pools comprising Superpool D2 were screened with FLB and 635-5', the primer pair that produce the 0.6 kb product when the *dpe1*-1 mutant DNA is present in the PCR template. A strong signal is visible in Pool 2400.

**Figure 4.4 Second round (Superpool) screening**

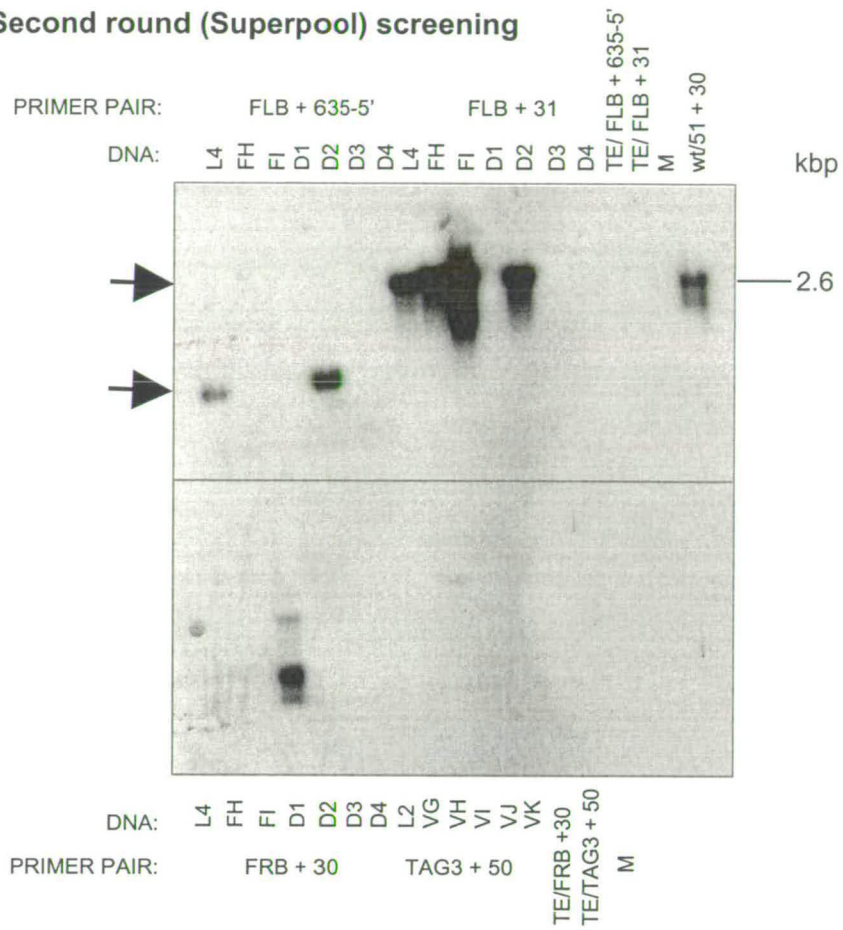



**Figure 4.5 Third round (Pool) screening**

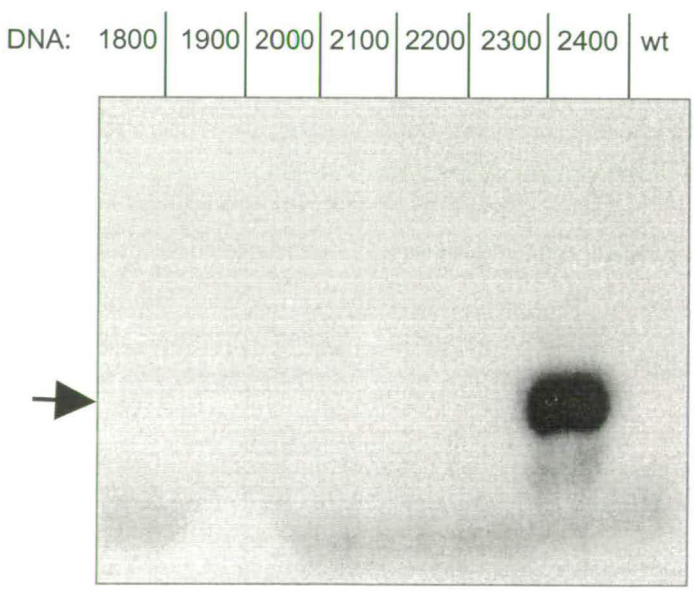


**Figure 4.6 'Subpool' screening**

Dupont pool 2400 was obtained as seed. One in every one hundred seeds should be contain a tagged D-enzyme gene. Seeds were germinated on sterile medium containing kanamycin. Kanamycin-resistant seedlings were transferred onto soil. Twelve trays each containing 60 plants were grown and at three weeks old, one leaf was excised from each plant and used to prepare 12 DNA templates (each corresponding to one tray).

These templates were subject to PCR using FLB and 635-5' primer pair. Products were resolved on an agarose gel and stained with ethidium bromide (UV fluorescent image).

The arrow indicates a 0.6 kb band where a *dpe1* mutation is present (trays 1, 3, 4, 9, 10 and 11).

**Figure 4.7 Further 'Subpool' screening**

Since the six plants containing *dpe1-1* mutations identified in Figure 4.6 were siblings and assumed to be identical, only two were investigated further. A second leaf was excised from the plants in trays 1 and 4 and used to prepare 6 DNA templates (each comprising 2 rows of 10 plants). These templates were subject to PCR using FLB and 635-5' primer pair. Products were resolved on an agarose gel and stained with ethidium bromide (UV fluorescent image). The arrow indicates a 0.6 kb band where a *dpe1*-1 mutant is present (tray 1 rows1 + 2 and tray 4 rows 3 + 4).

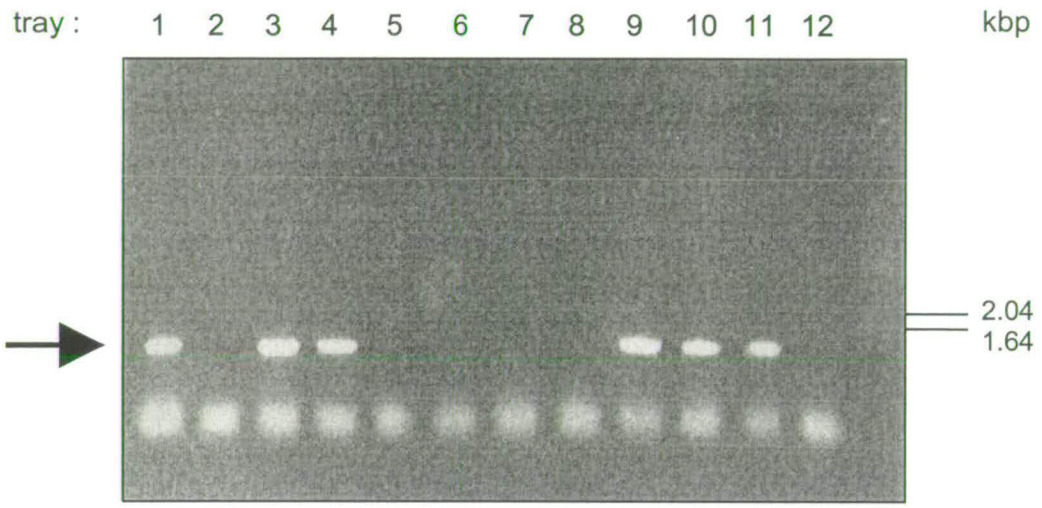



**Figure 4.6 'Subpool' screening**

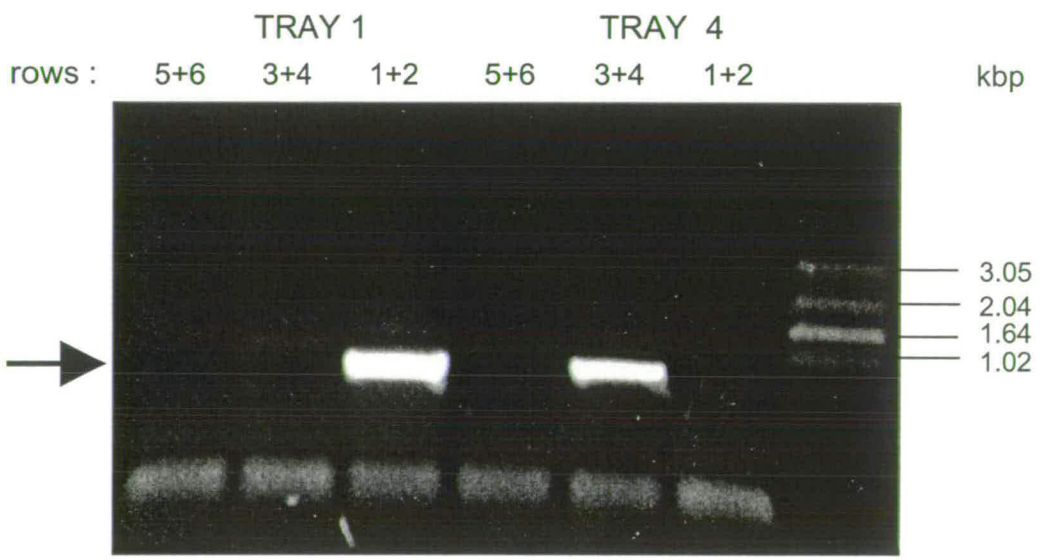



**Figure 4.7 Further subpool screening**

**Figure 4.8 Screening of individual plants**

A third leaf was excised from the plants in tray 4 rows 3 and 4. These 20 leaves were each used to prepare a crude DNA template for PCR on individual plants.

These templates were subject to PCR using FLB and 635-5' primer pair. Products were resolved on an agarose gel and stained with ethidium bromide (UV fluorescent image).

The arrow indicates the 0.6 kb product indicating that plant 4.3H contains the *dpe1*-1 mutation.

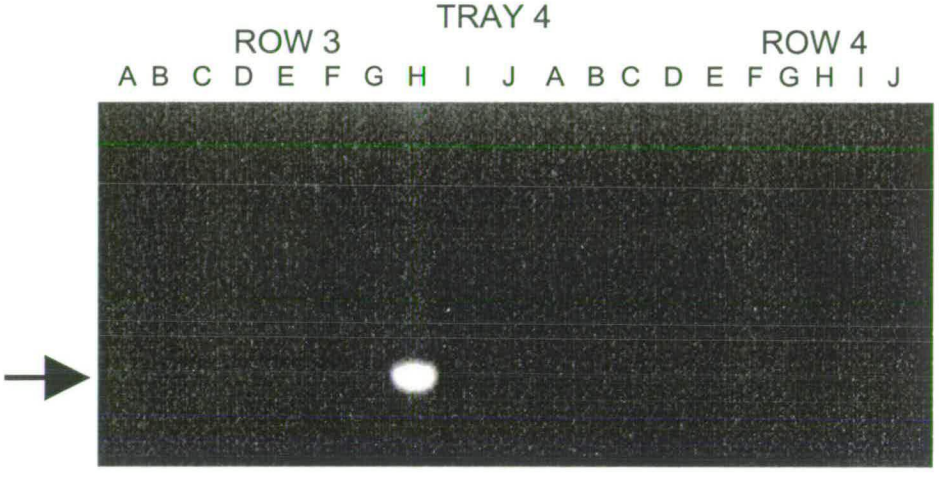



**Figure 4.8 Screening of individual plants**

### 4.3 Isolation of the homozygous mutant plant

It is possible to determine whether the mutant plants are heterozygous or homozygous by PCR if the sequence on the other side of the T-DNA insert is known. At the time of screening, the 5'end of the D-enzyme sequence was the region on the other side of the insert, and was unknown. Therefore homozygous mutants were identified from the progeny of mutant plant 4.3H by western analysis. Proteins were blotted onto membranes and probed with antibodies raised against recombinant *Arabidopsis* D-enzyme. Out of 18 mutant progeny screened, 6 showed no immuno-reactive D-enzyme band. From these homozygous mutants A1, A4, A5, B4, C5 and D1 and heterozygote A2 (Figure 4.9), seed was collected. Further analysis has been performed on progeny of homozygous mutants A5 and D1.

It is interesting to note that the heterozygous mutant shows an immuno-reactive band of half the intensity of that of the wild-type suggesting that levels of the protein may be gene dose-dependent.

**Figure 4.9 Isolation of the homozygous mutant plant**

Homozygous plants were identified from the progeny of mutant plant 4.3H by western analysis. Total proteins (10 μg) were separated by SDS PAGE, blotted onto membrane and probed with antibodies raised against recombinant *Arabidopsis* D-enzyme. Out of 18 mutant progeny screened, 6 showed no immuno-reactive D-enzyme band. Homozygous mutants A1, A4, A5, B4, C5 and D1 are shown along with heterozygote A2 and wild type. A long exposure is provided to illustrate equal loading with respect to the non-specific bands.

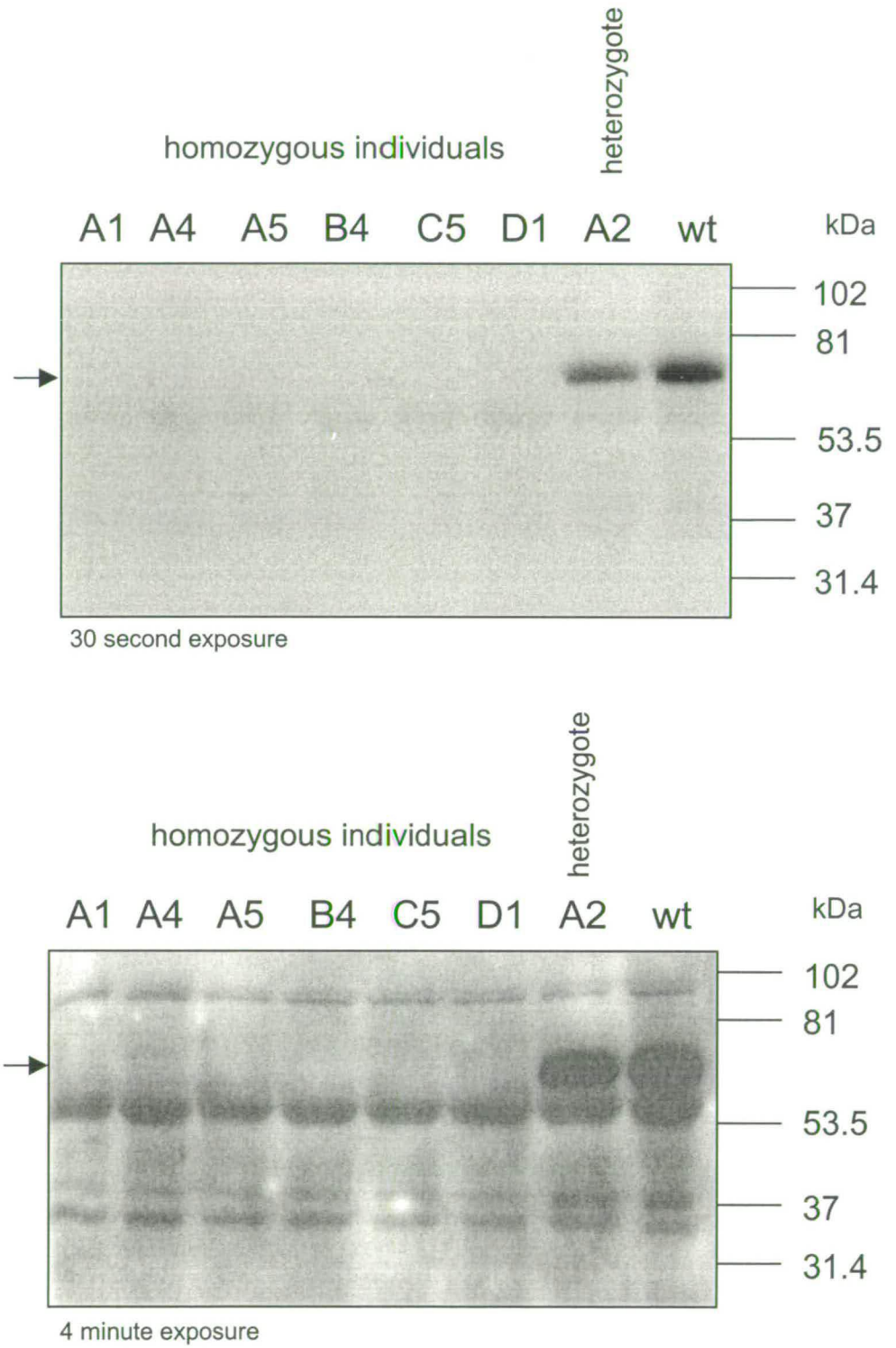



**Figure 4.9 Isolation of the homozygous mutant plant.**

### 4.4 Site and arrangement of T-DNA insertion in the D-enzyme gene

The upstream and downstream junctions of the T-DNA insertion in the D-enzyme gene were determined by directly sequencing the PCR products. Figure 4.10 summarises the isolation of mutant and indicates the position of insertion. The junction sequences and precise insertion site are shown in Figure 4.11.

The T-DNA insertion was located at 368 nucleotides downstream of the ATG initiation codon within exon sequence, a location that is expected to generate a null allele. A deletion of 24 bases of the left border of the T-DNA had occurred upon insertion of the element. It was not possible to amplify the junction between FRB and 5'END, a primer that anneals to the 5'end of the D-enzyme sequence. PCR with FLB and 5'END produced a 0.7 kb fragment which sequencing showed to contain the junction between the T-DNA and the D-enzyme gene. A deletion of several bases and a small amount of rearrangement had occurred at the junction. From this information the T-DNA insertion appears to contain a minimum of two elements in inverted orientation with the left borders at each end.

### 4.5 Determination of the number and orientation of inserts in mutant

DNA was extracted from *dpe1-1* mutant plants, digested with *Eco RI* and the fragments separated by electrophoresis. DNA was transferred onto nylon membrane and probed first with a PCR derived D-enzyme partial genomic sequence, then stripped and probed with a kanamycin resistance gene sequence (shown in Figure 4.12, p93). The total length of each T-DNA repeat is 16901 kb. *Eco RI* cuts three times in the T-DNA at 78, 3011 and 10 284. Internal fragments are thus expected to be 7.3 kb and 2.9 kb (non-hybridising). Possible internal fragments, if T-DNA is arranged in inverted repeats, are thus 13.2 kb for adjacent sequences facing in opposite directions to each other and 6.6 kb for adjacent sequences facing in the same direction as each other. *Eco RI* does not cut within the genomic sequence, but about 7.5 kb downstream of the ATG. A 7.8 kb fragment hybridises with a genomic D-enzyme probe.

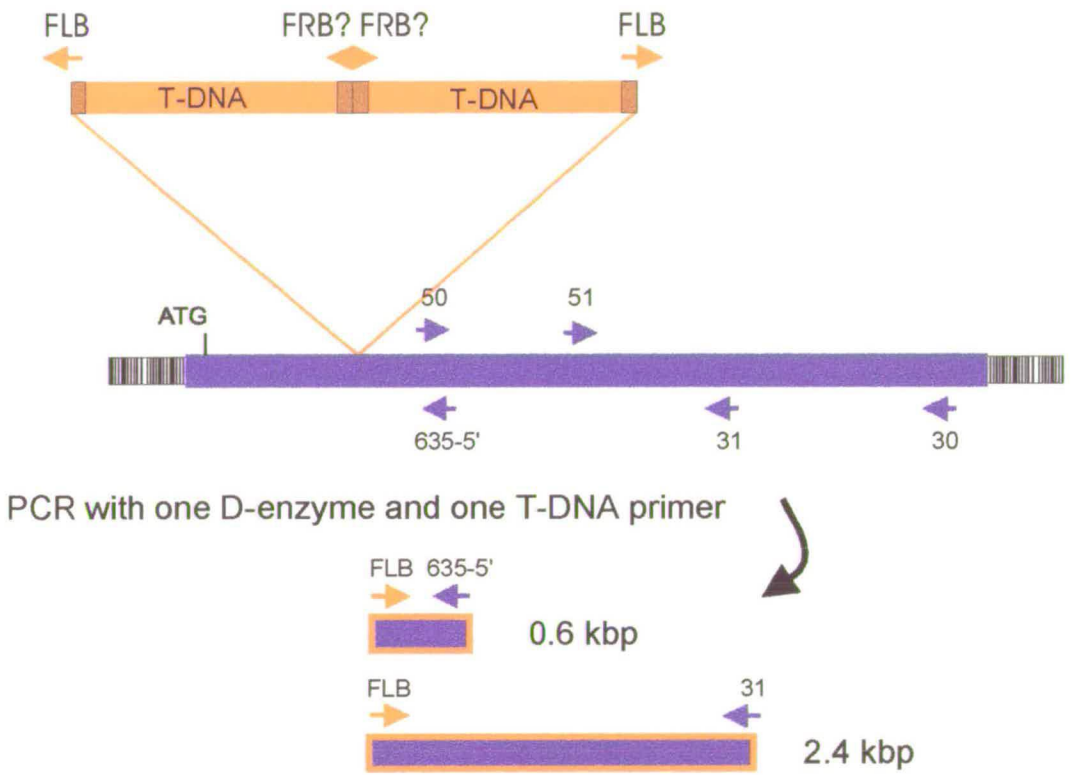


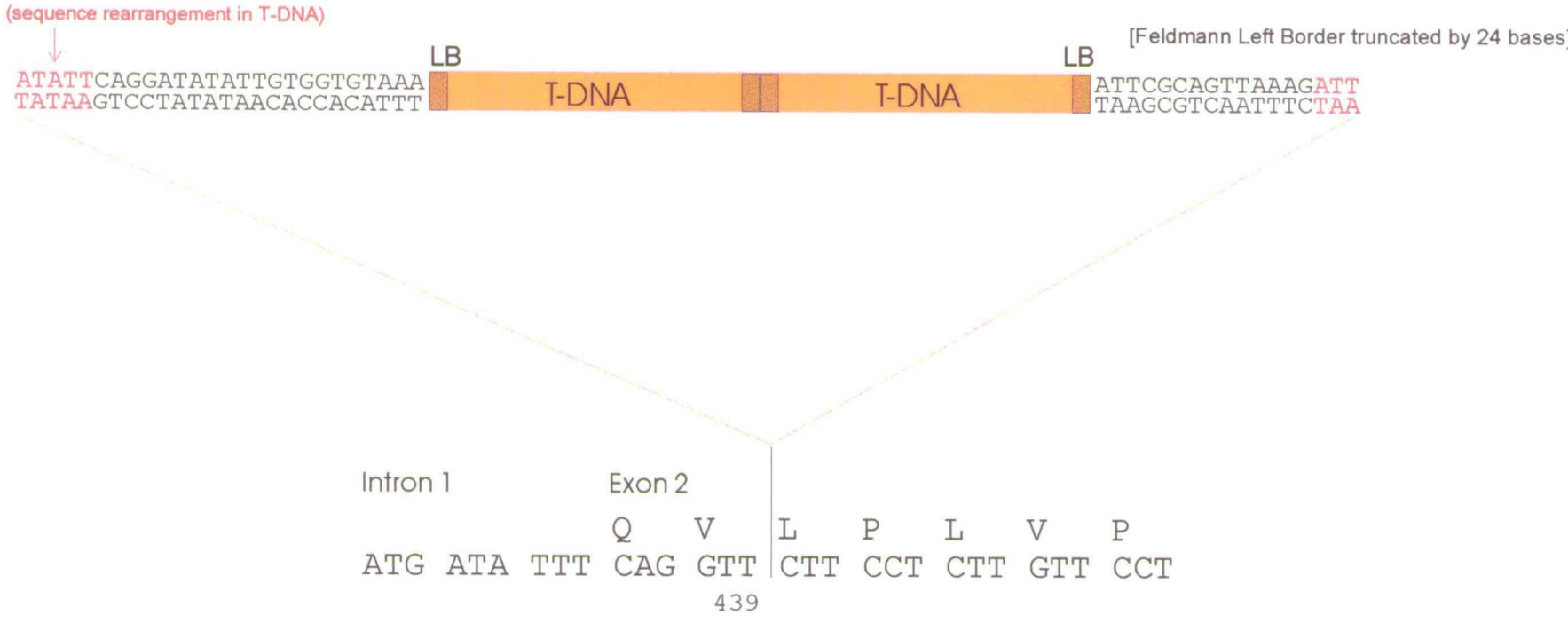

**Figure 4.10 Isolation of T-DNA insertion in D-enzyme 1 gene, *dpe1-1***

A physical map of the DPE1 D-enzyme gene is presented along with the relative positions of the FLB and gene specific primers used in the screening. PCR products. Only the left border T-DNA oligo would amplify DPE1 sequences on either side of the insertion . The T-DNA element is not drawn to scale and deletions and rearrangements which may have ocurred are not shown. There are probably three or four tandem T-DNA copies at this site (discussed in section 4.4).

**Figure 4.11 Sequence of the insertion site of the *dpe1-1* mutation**

PCR with FLB and and 635-5' produced a 0.6 kb fragment , and PCR with FLB and 5'END produced a 0.7 kb fragment. These fragments contained the junctions between the T-DNA and D-enzyme gene. The two fragments were sequenced using FLB plus 635-5' and 5'END primers respectively. The sequence of the insertion site is shown. Red type represents sequence which has been re-arranged as a result of the insertion event.

**Figure 4.11 Sequence of the *dpe1-1* mutation.**



The hybridisation pattern seen in this blot could be explained by the presence of inverted repeats. However, due to the possibility of molecular rearrangement in the T-DNA it is not certain what restriction sites are present in the sequence. The presence of the same pattern in all five independent plants (and also in a similar experiment where the DNA was cut with *Xho I*, data not shown) suggests that it is unlikely that the *dpe1-1* plant contains any additional T-DNA insertion at another site independent from that in the D-enzyme gene.

A sibling mutant plant (A3) was crossed with wild-type and numbers of kanamycin-sensitive plants resulting from the F2 seed were determined. A homozygous plant containing one T-DNA insert in a single gene, crossed with wild-type would be expected to give an F2 generation of 3:1 kanamycin resistant : kanamycin sensitive plants. Three crosses gave an F2 generation with a 15:1 ratio, the result expected for two independent insertion events. This result has not been confirmed with the mutant subject of this study.

### 4.6 Conclusion

Using a PCR-based screen of 19 000 T-DNA-containing *Arabidopsis* plant lines, one plant line was found in which the D-enzyme gene was disrupted by a T-DNA insertion. The T-DNA insertion was located at 368 nucleotides downstream of the ATG initiation codon within exon sequence. A plant line homozygous for the D-enzyme mutation was isolated by western blotting since it contained no detectable D-enzyme protein. Southern blots were performed on DNA from mutant plants that had been digested with restriction enzymes. When probed with the kanamycin resistance gene sequence (which anneals to T-DNA insertion) and then D-enzyme sequence, the fragment pattern in a number of independent plants was the same, a result of low probability if there was an additional insertion. However this experiment needs to be repeated with enzymes which do not cleave between the T-DNA and D-enzyme. Crossing a sibling of the mutant plant with wild-type and determining the number of kanamycin sensitive progeny suggested that the plant might contain an additional T-DNA insertion not associated with that disrupting the D-enzyme gene, although this has yet to be confirmed.

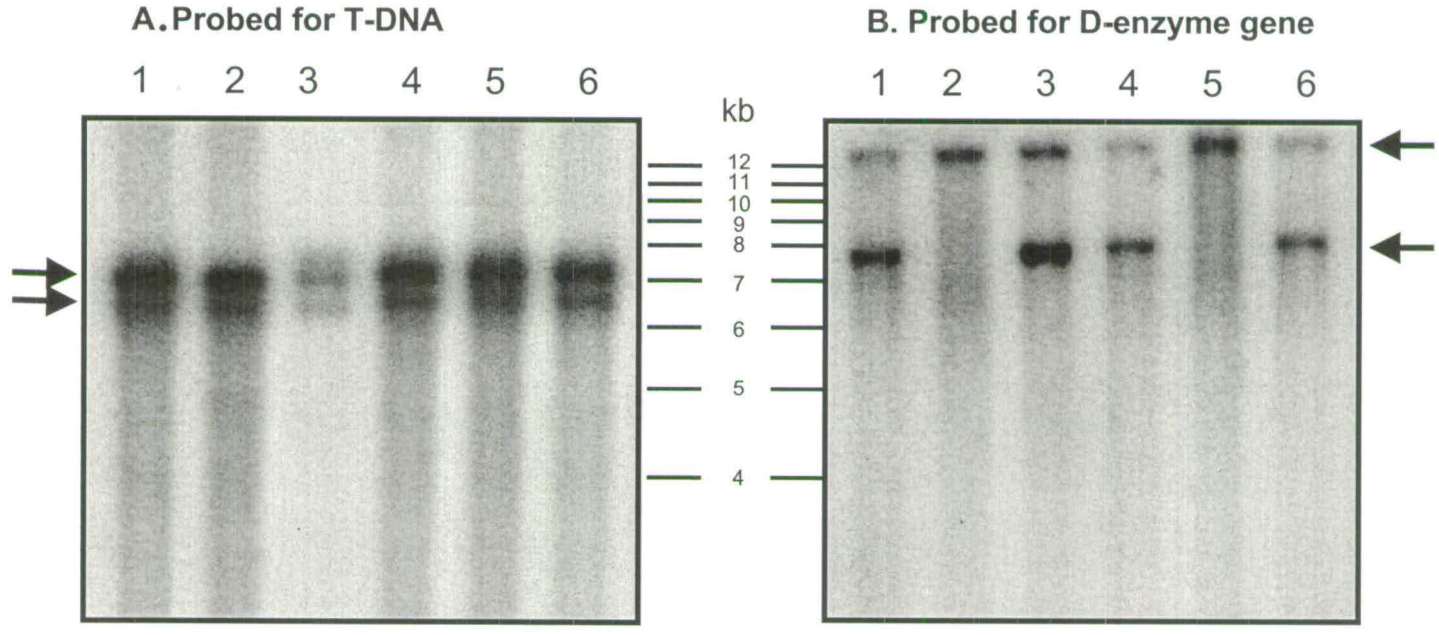



**Figure 4.12 Analysis of restriction enzyme fragment patterns in independent mutant plants in order to deduce arrangement of the T-DNA elements.**

Southern blot of *Eco RI* digested genomic DNA from six independent *dpe-1* mutant plants

(Lanes 1, 3, 4 and 6 were homozygous 2 and 5 were heterozygous for the *dpe1-1* mutation.)

# Chapter 5

# Analysis of D-enzyme Gene Expression in Wild-type and Mutant plants

### 5.1 Introduction and Aims

The analysis of mutants deficient in various enzymes has contributed much to current understanding of starch metabolism. No mutant for D-enzyme had been isolated from any higher plant and since the phenotypic changes resulting from a lack of D-enzyme were unknown it was difficult to devise a phenotypic screen for a mutant. Consequently, the study of D-enzyme lags behind that of other starch metabolic enzymes and it is still uncertain what role D-enzyme plays *in vivo*.

Chapters 3 and 4 describe the preparation of experimental tools to be used in further investigations. This chapter aims to describe the use of these tools in some characterisation of D-enzyme expression in *Arabidopsis thaliana* and preliminary characterisation of the D-enzyme null mutant plant.

D-enzyme had been shown to be expressed in leaves of *Arabidopsis*. In potato, where expression had been investigated more thoroughly, D-enzyme transcripts were detected in leaves, petioles, stems, roots and stolons, being most abundant in developing and mature tubers. In potato, mRNA accumulates under circumstances where starch biosynthesis is most active and declines in amount under conditions where starch is broken down (cold stored tubers and sprouting tubers) suggesting that regulation of the gene is under developmental and metabolic control at transcriptional level. D-enzyme protein expression, however, remained constant, suggesting post-transcriptional regulation of gene expression. It was of interest to determine the expression patterns of D-enzyme in *Arabidopsis* over a day/night period within the leaf and also during the development of the plant. This has yet to be investigated in a system that does not accumulate starch in a storage organ. This might offer further information as to when its action is required by the plant and shed some light on its role in metabolism.

At least 80 % of D-enzyme activity in the *Arabidopsis* leaf is confined to the chloroplast. It is unknown whether D-enzyme is distributed throughout the chloroplast stroma or confined to particular regions. It is unknown too whether it is located at the starch granule surface or whether it becomes trapped between layers of

starch during the construction of the granule. This information might support the role of D-enzyme with respect to one of the proposed models of debranching enzyme activity.

Once a null mutant had been isolated, the phenotype resulting from lack of D-enzyme could be investigated. It was important initially however to establish that other enzymes of starch metabolism in the mutant have normal levels of activity, so that any phenotypic changes can be attributed to the lack of D-enzyme alone. The aim of this work was to determine the effect that loss of D-enzyme has on plant growth, on the plant's ability to metabolise starch and on the starch itself.

## 5.2 Expression of D-enzyme gene in *Arabidopsis* plants

### 5.2.1 Analysis of D-enzyme expression in different organs

Control of D-enzyme gene expression at the protein level during the development of wild-type *Arabidopsis* plants was analysed by western blotting. Soluble proteins were extracted from a variety of tissues and probed with D-enzyme antisera (Figure 5.1). Under normal conditions, D-enzyme protein is detectable throughout the plant and throughout development with the exception of roots. However, D-enzyme is found to be well expressed in roots that had been grown in sterile culture on a medium containing 3% sucrose and also in roots which had greened near the top due to exposure to light (data not shown). All green tissues including leaves, stem, stigmas and siliques show equal band intensities of D-enzyme protein, as do flower buds and flowers (of which sepals and stigmas are green). Interestingly, D-enzyme is also expressed in petals. The amount of D-enzyme declines in senescing tissues (in 25% yellow leaves, 75% yellow leaves, 50% yellow and 100% yellow siliques).

### 5.2.2 Analysis of D-enzyme expression through day/night cycle

Control of D-enzyme gene expression at the protein level during a day/night cycle of wild-type *Arabidopsis* plants (grown with a 12 h photoperiod) was analysed by western blotting. Soluble proteins were extracted from leaf tissue collected at intervals during 24 h and probed with D-enzyme antisera (Figure 5.2). D-enzyme protein is present at a constant level throughout the day and night.

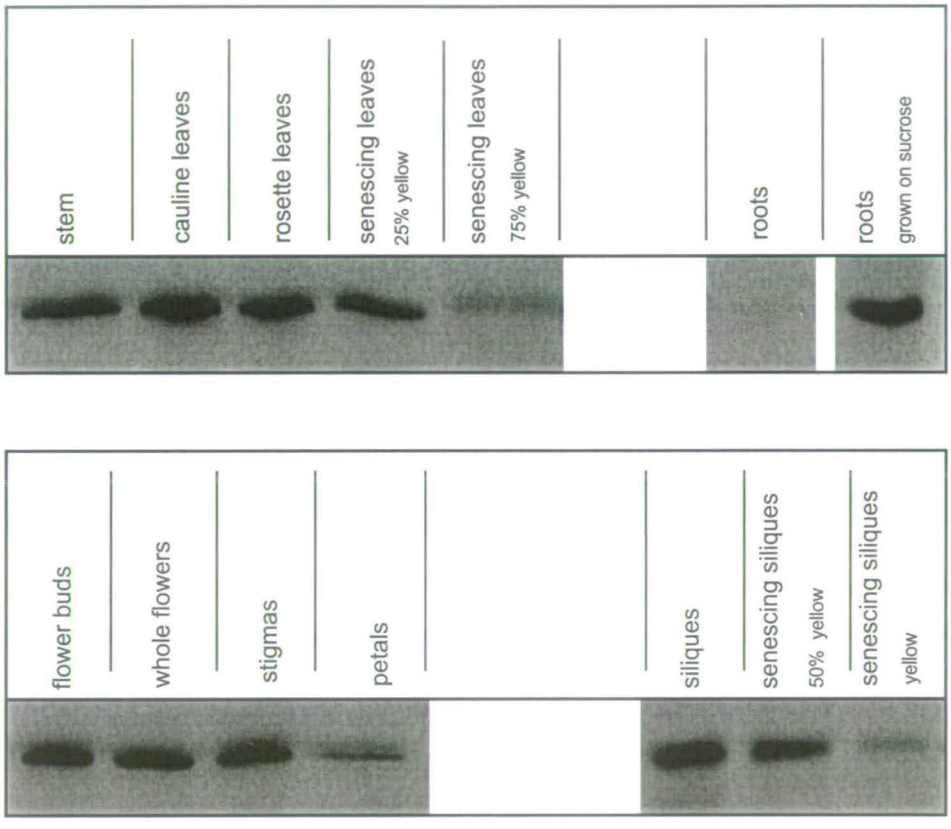



**Figure 5.1 Organ specific expression of D-enzyme in wild-type plants**

Total soluble protein (10μg) from organs of *Arabidopsis* was separated by SDS PAGE, blotted onto membrane and probed with antibodies raised against recombinant *Arabidopsis* D-enzyme.

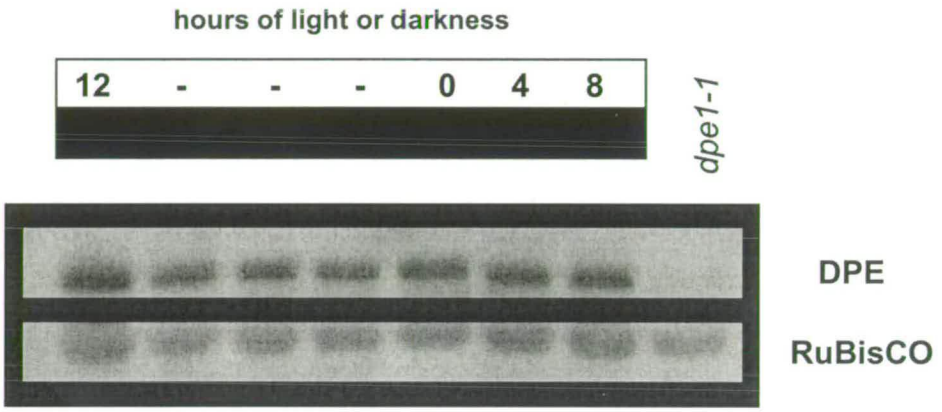



**Figure 5.2 Expression of D-enzyme in wild-type plants over a 24-hour period**

Total soluble protein (10 μg) from leaves of wild-type plants harvested over a 24 h period was separated by SDS PAGE, blotted onto membrane and probed with antibodies raised against recombinant *Arabidopsis* D-enzyme. Non-specific binding of antibody to RuBisCO large subunit seen in longer exposure included for comparison of equal protein loading.

### 5.2.3 Sub-cellular localisation of D-enzyme

Leaf tissue was harvested from plants mid-photoperiod, resin-embedded and sections were prepared for analysis by Transmission Electron Microscopy. Sections were labelled using D-enzyme antisera followed by gold-conjugated second antibody. The TEM sections are shown in Figure 5.3.

Leaves from a *dpe1-1* plant were also processed as a control, to give an indication of background staining. Due to the non-specific binding of the antibody to RuBisCO there is some label in the thylakoid membranes. There is a small amount of labelling of the starch granules of the mutant (section B) which is thought to be due to a slight affinity of the serum for starch. This has been reported in the work of others as due to a component of the adjuvent used in inoculation.

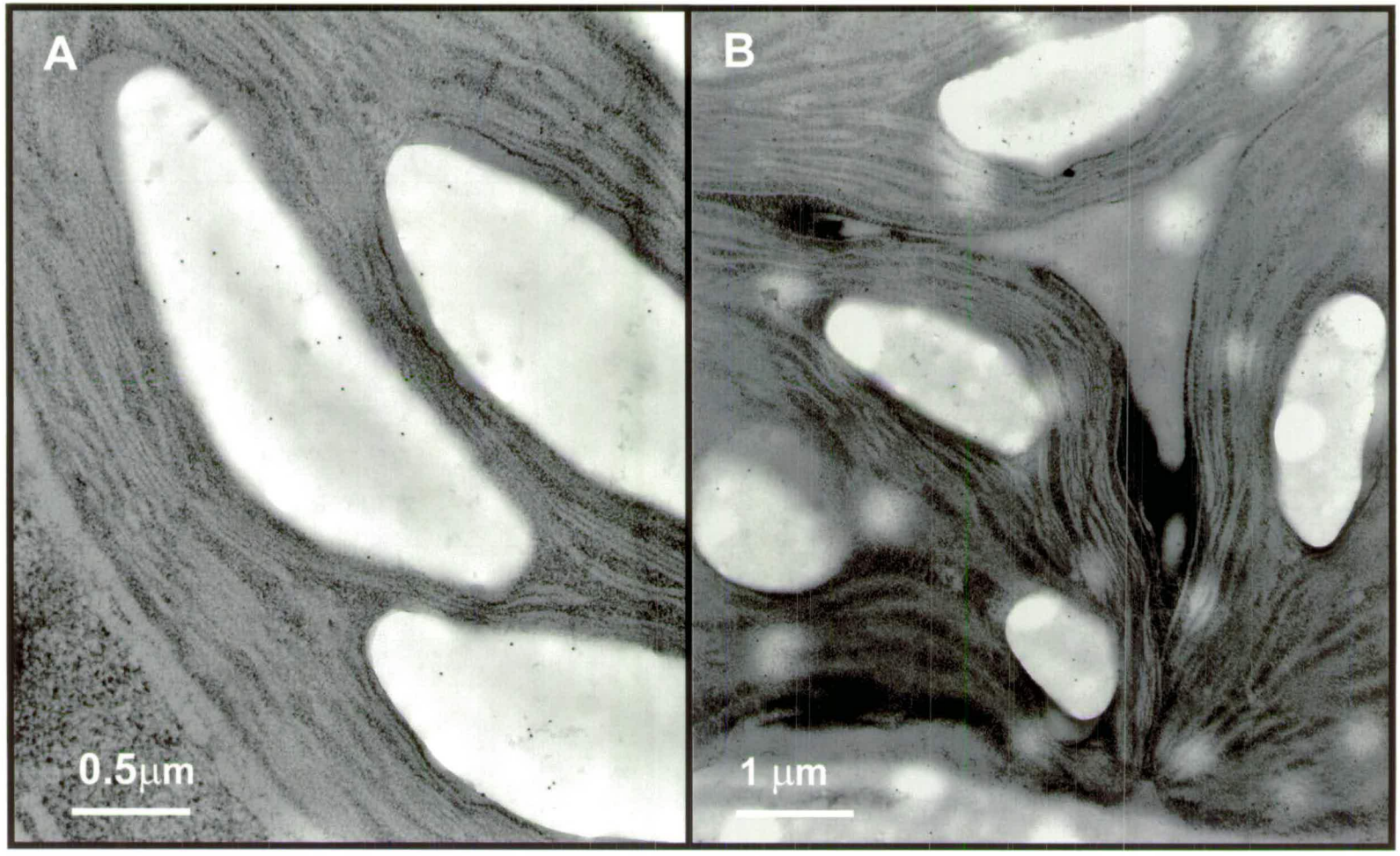
However, the wild-type tissue (section A) shows significant staining on the starch granules in addition to the background staining determined from mutant. Insignificant labelling was seen outside the chloroplast (data not shown).

**Figure 5.3 The subcellular localisation of D-enzyme**

Immuno-gold labelled leaf tissue sections analysed using transmission electron microscopy (performed at the Electron Microscopy Suite, University of Edinburgh).

A. Wild-type tissue shows significant staining in the starch granules
B. *dpe1-1* tissue shows the level of background staining: non-specific binding of antibodies to RuBisCO (small amount of labelling in thylakoid membranes) and to the starch.

Note that the scale of the photograph in A is twice that of B to demonstrate these points. A formavar grid was used to support the sections since starch granules in thin sections fall out easily. White patches in the pictures are holes in the formavar grid and not the related to the structure of the tissue.


A
0.5μm
B
1 μm

## 5.3 Effect of D-enzyme gene disruption on protein expression and activity

### 5.3.1 Analysis of D-enzyme protein expression in *dpe1-1* mutant plants

D-enzyme expression in the mutant plant *dpe1-1* was analysed by western blotting. Soluble proteins were extracted from leaf tissue from mutant and wild-type plants and probed with anti-D-enzyme antisera (Figure 5.4). Even after an exposure time of 4 minutes (over-exposure) which is sufficient to visualise even small amounts of non-specific hybridising, no trace of D-enzyme expression is detected in the leaves of the plant containing a T-DNA insertion in the D-enzyme gene.

### 5.3.2 Biochemical assay of D-enzyme activity

The activity of D-enzyme was measured in crude extracts of leaves of *dpe1-1* and wild-type plants, using maltotriose as a substrate. Wild-type activity (283 ± 15 nmol $min^{-1}$ $g^{-1}$ fresh weight) was in good agreement with that previously published for D-enzyme in *Arabidopsis* leaves (Lin and Preiss, 1988; Zeeman *et al.*, 1998). The D-enzyme activity in the mutant was effectively zero (9 ±10 nmol $min^{-1}$ $g^{-1}$ fresh weight). Maltase can potentially interfere with the assay for D-enzyme as the substrate specificity of maltases can be broad (Sun *et al.*, 1995). However, the low activity of maltase compared with D-enzyme (Table 5.1) and the difference in pH requirements of D-enzyme and maltase (which have optimum activity at a pH of 6.8 and 5.2 respectively) make it likely that an error in the D-enzyme activity should be small.

## 5.4 Effect of D-enzyme disruption on other genes of starch metabolism

### 5.41 Biochemical assay of starch metabolising enzymes

The activities of starch-degrading and starch-synthesising enzymes were measured in crude extracts of leaves of *dpe1-1* and wild-type plants. This work was carried out in collaboration with Professor Alison Smith and Dr. Sam Zeeman at the John Innes Centre, Norwich.

α-amylase was measured using starch azure and was saturated with β-amylase (Doehlert and Duke, 1983). β-amylase was assayed by measuring the production of maltose from potato starch. Maltase was measured with maltose as a substrate.

The activities measured are summarised in Table 5.1.

**Table 5.1. Comparison of the maximum catalytic activities of starch metabolising enzymes in crude extracts of wild-type and mutant *dpe1-1* *Arabidopsis***

| Enzyme | Activity (nmol $min^{-1}$ $g^{-1}$ fresh weight) | | Statistically significant differences | |
|---|---|---|---|---|
| | | | of means | of distributions |
| | Wild-type | Mutant | Student's *t*-test | using standard deviations |
| α-amylase | 7.03 ± 0.86 | 7.97± 1.89 | $p < 0.661$ | |
| β-amylase | 1234 ± 142 | 2167 ± 357 | **$p < 0.041$** | |
| Pullulanase | 50.4 ± 4.0 | 50.5 ± 3.3 | $p < 0.987$ | |
| Maltase | 39.0 ± 3.2 | 23.8 ± 6.0 | **$p < 0.055$** | |
| D-enzyme | 283 ± 15 | 9 ± 10 | **$p < 0.001$** | **$p < 0.001$** |
| Starch phosphorylase | 76.5 ± 7.5 | 102.3 ± 3.3 | $p < 0.029$ | |
| Total starch synthase | 119 ± 21 | 90 ± 25 | $p < 0.391$ | |
| Starch branching enzyme† | 4.44 ± 0.64 | 4.22 ± 0.85 | $p < 0.845$ | |

The values are the means of measurements made on five independent extracts ± SEM

† activity was measured as the stimulation of the incorporation of $^{14}C$ from [$^{14}C$] glucose-1-phosphate by phosphorylase into glucan as μmol $min^{-1}$ $g^{-1}$ fresh weight (Smith, 1988).

A very highly significant reduction in D-enzyme activity was found in the *dpe1-1* mutant using both the Student's *t*-test and a comparison of data distributions using multiple standard deviations. The Student's *t*-test also showed the activities of β-amylase and starch phosphorylase to be significantly higher in the mutant than in the wild-type. A slightly significant reduction in the activity of maltase was also found in the leaves of the mutant.

The *t*-test showed the above differences to be significant as it compares the overlap of means rather than the overlap of data distributions, which is a more rigorous analysis.

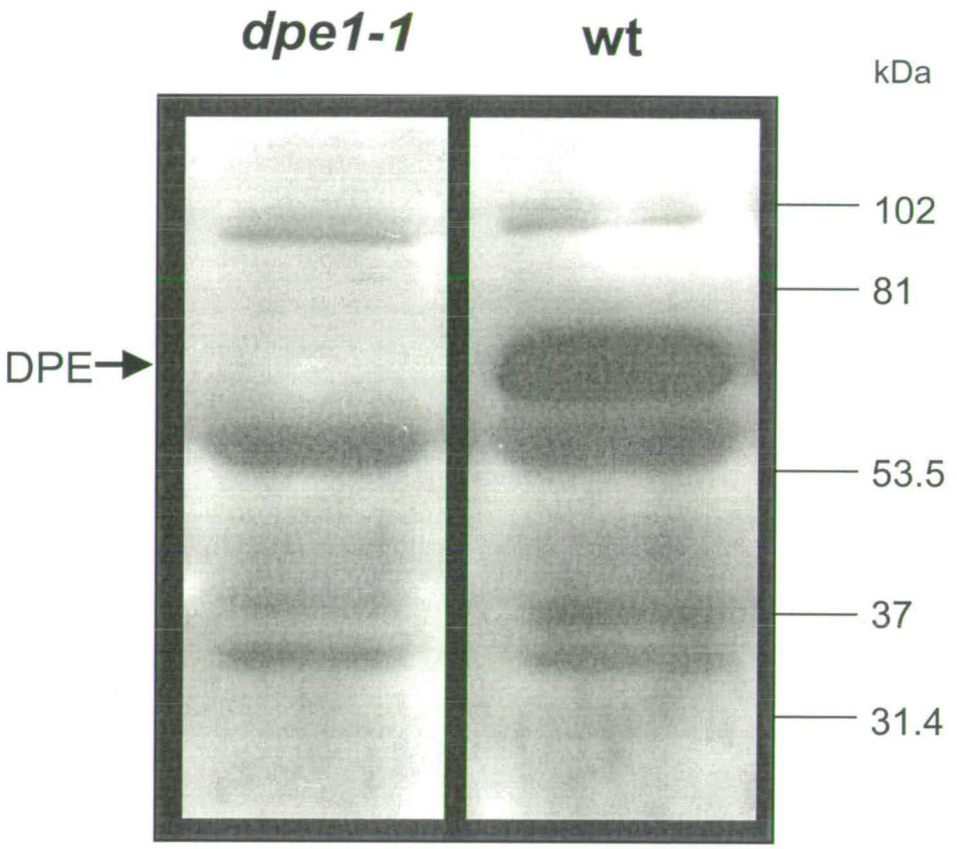



**Figure 5.4 Effect of D-enzyme gene disruption on protein expression**

Total soluble protein (10 μg) from leaves of mutant and wild-type plants was separated by SDS PAGE, blotted onto membrane and probed with antibodies raised against recombinant *Arabidopsis* D-enzyme. This is an overexposure (4 min) to illustrate that there is no trace of D-enzyme in the *dpe1-1* plants.

### 5.42 Native PAGE 'activity staining'

Native PAGE was used to separate enzymes of starch degradation. This technique is not quantitative but is complementary to that of biochemical assay since it allows the separation of different isoforms of each enzyme.

Starch phosphorylase isoforms were separated on a gel containing glycogen, for which the plastidic and cytosolic isoforms differ in their affinity (Steup, 1990). The band corresponding to the chloroplastic isoform (P2) in extracts of wild-type leaves was very faint (Figure 5.5). This is consistent with the measurements of plastidic starch phosphorylase activity made by Lin *et al.* (1988) in which only 4% of the total phosphorylase activity in wild-type *Arabidopsis* leaves was in the chloroplast. There was no detectable change in the starch phosphorylase activity of the D-enzyme mutant.

## 5.5 Effect of absence of D-enzyme on plant growth and development

### 5.51 Seed germination

The germination time of *dpe1-1* seed on soil and in sterile culture was the same as wild-type Ws seed (not shown).

### 5.52 Plant growth

The effect of the mutation in the D-enzyme gene on the growth rate of the plants was investigated. Plants were germinated and grown on soil in a growth cabinet under a 10 h photoperiod and their growth was recorded at weekly intervals. Figure 5.6 plots the mean plant fresh weight (excluding roots) from one week after germination to flowering. Each value is the mean weight of 10 plants. The data are also recorded in Table 5.2 since the data of weeks one and two after germination is not clear due to the scale of the graph.

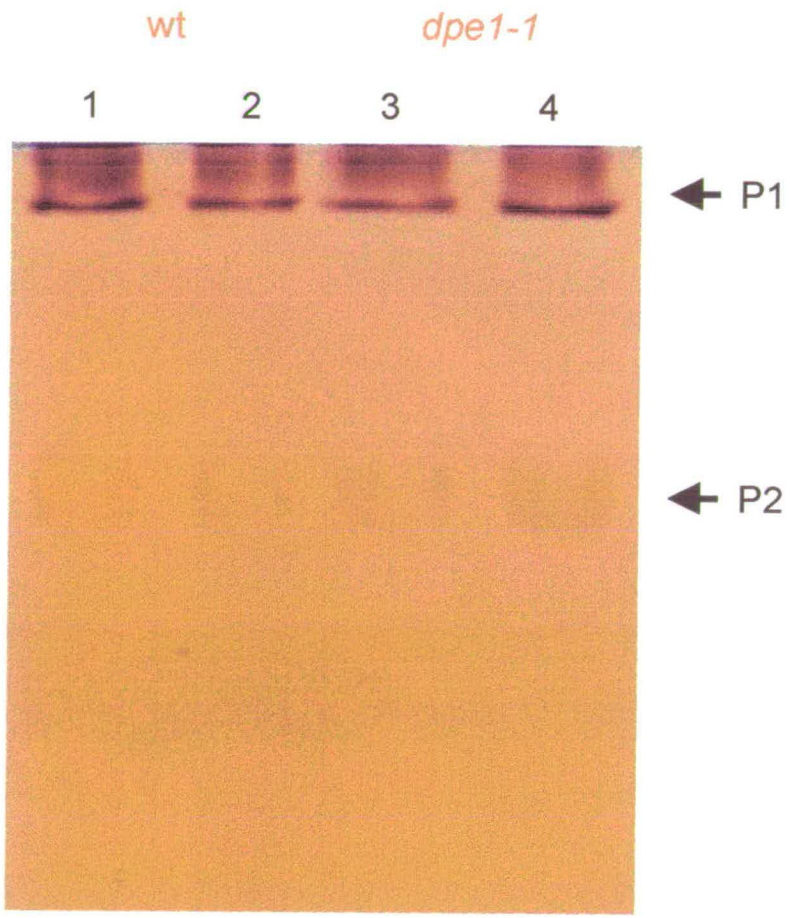



**Figure 5.5 Native PAGE of starch phosphorylase isoforms.**

Soluble proteins from crude extracts of leaves were subjected to native PAGE in gels containing glycogen, then incubated overnight at pH 6.6 with glucose-1-phosphate to reveal starch phosphorylases.

**Table 5.2 Mean weight of wild-type and mutant *Arabidopsis* plants from germination to flowering**

| weeks after germination | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| wt (mg) | 1.2 | 12 ± 0.8 | 44.7 ± 4.4 | 173.5 ± 13.8 | 741.8 ± 60.8 | 1138.9 ± 96.8 | 1046.9 ± 119.5 |
| *dpe1-1* (mg) | 0.7 | 6.8 ± 0.9 | 22 ± 1.1 | 130 ± 7.8 | 491.5 ± 33.9 | 932.2 ± 51.8 | 926.5 ± 97 |

After the first few weeks of growth, the fresh weight of the mutant plant lagged behind the wild-type by 40-50%. At four or five weeks the weight of the mutant lagged behind the wild-type by about 30%. As the plants approached flowering the difference between them decreased further although at the point of flowering the mutant plants were still 18% lighter than the wild-type. After flowering the weights of the two are similar within the variation between individual plants.

A digital image of ten plants for each time point was also recorded using a scanner. Figure 5.7 shows a representative sample of *dpe1-1* and wild-type plants from each time-point. For the first four weeks, the leaves are clearly larger in the wild-type plants than in the mutant. At five weeks the leaves of the mutant reach an equivalent size to the wild-type and the rosettes appear to have a similar diameter. However, the leaves of the mutant are lighter in colour and appear thinner or less substantial. It was of interest whether the total number of leaves or the photosynthetic area was different in the mutant. The mean total photosynthetic area of each plant and the number of leaves were calculated from these images. Due to the nature of the method of calculation, no error bars are included. Figure 5.8 plots mean leaf area per plant from one week after germination to the initiation of flowering. In week 1 the leaf areas were mostly those of cotyledons; the mutant had 20% less cotyledon area. When the true leaves emerge by week 2 the mutant has almost 50% less leaf area than the wild-type. This deficit in leaf area decreases as the plants grow over the five weeks to 20% less than the wild-type by week 5. Figure 5.9 plots the average number of leaves per plant from one week after germination to the initiation of flowering. The mutant plant lagged behind the wild-type plant by approximately one

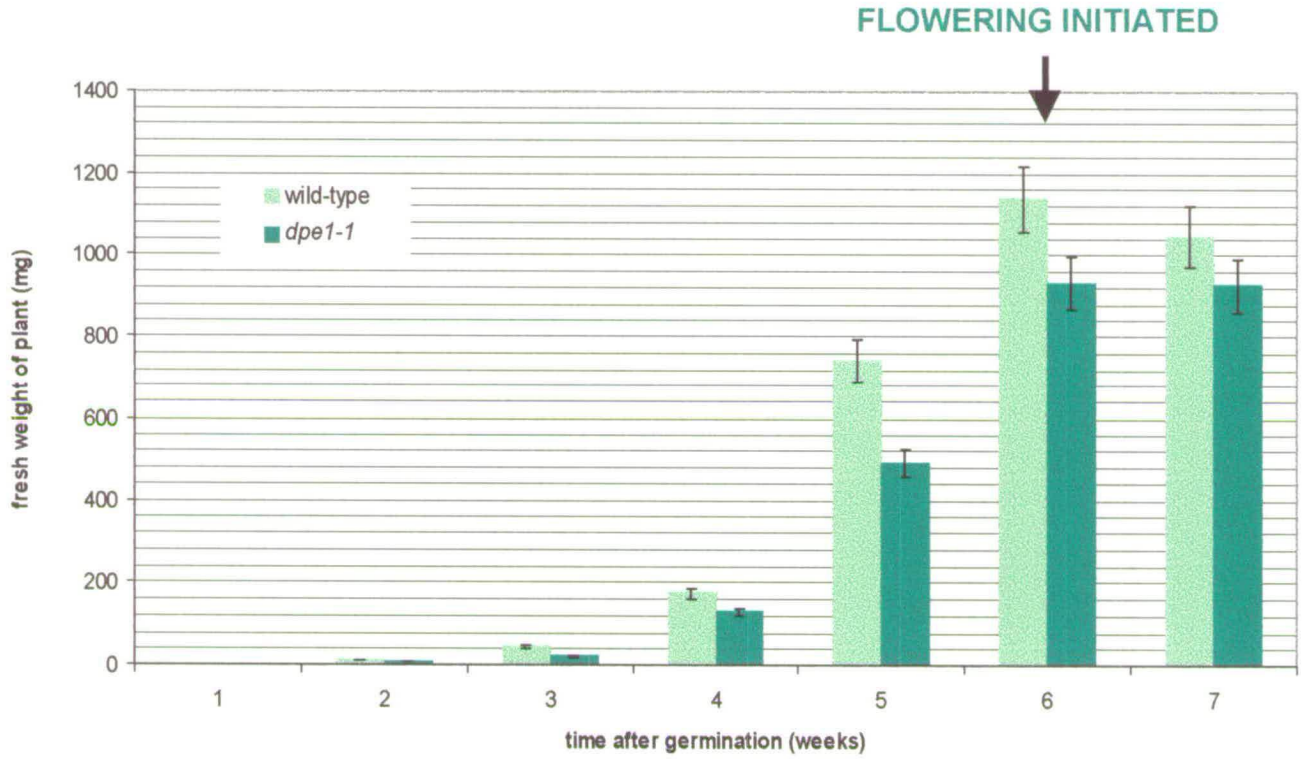



**Figure 5.6 Comparison of weight of D-enzyme and wild-type plants from germination**

After germination on soil, individual plants were weighed at weekly intervals until the plants flowered and the leaves began to senesce (week 7). The mean weight of 10 plants is plotted.

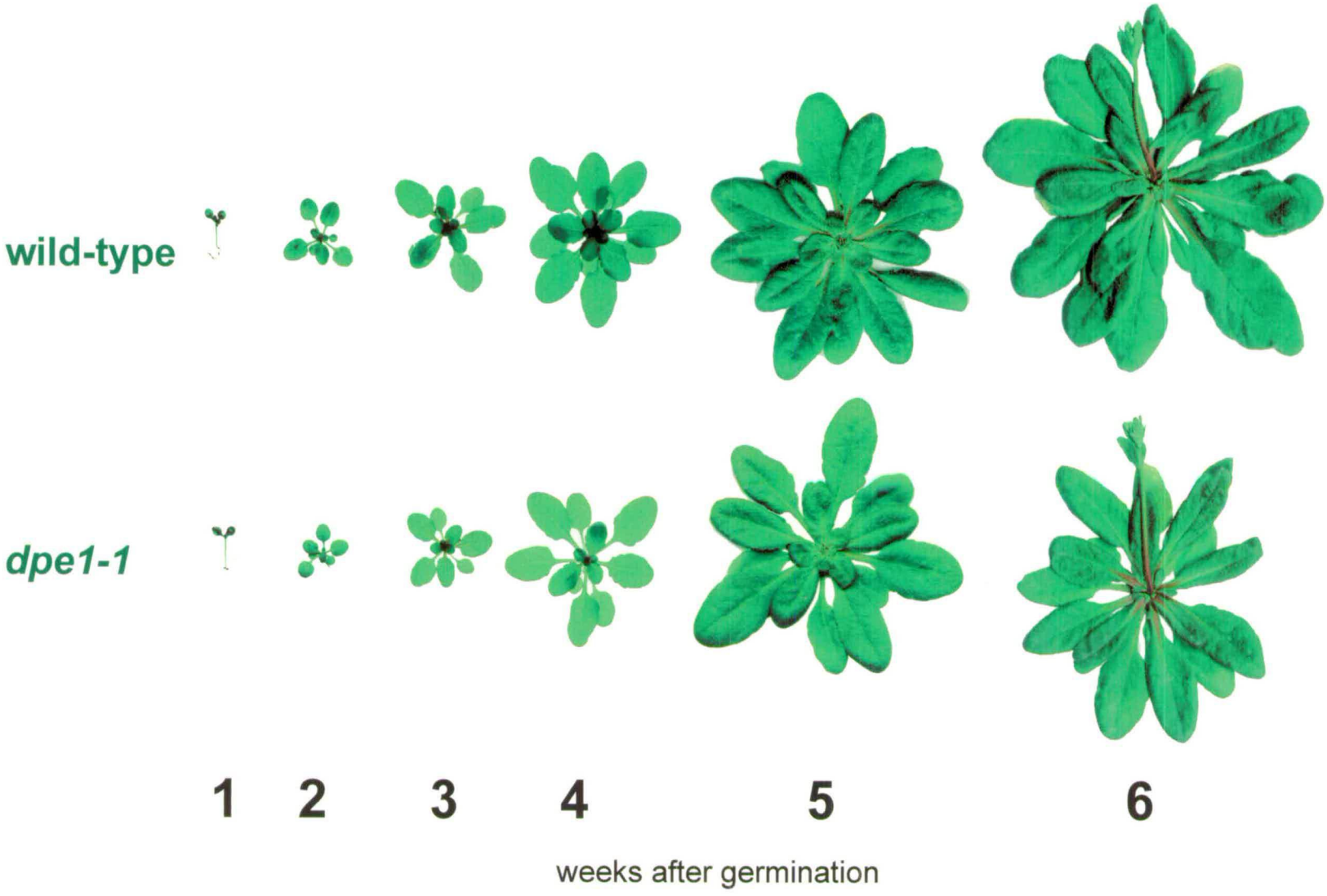



**Figure 5.7 Relative growth rate of D-enzyme mutant and wild-type plants from germination to flowering**

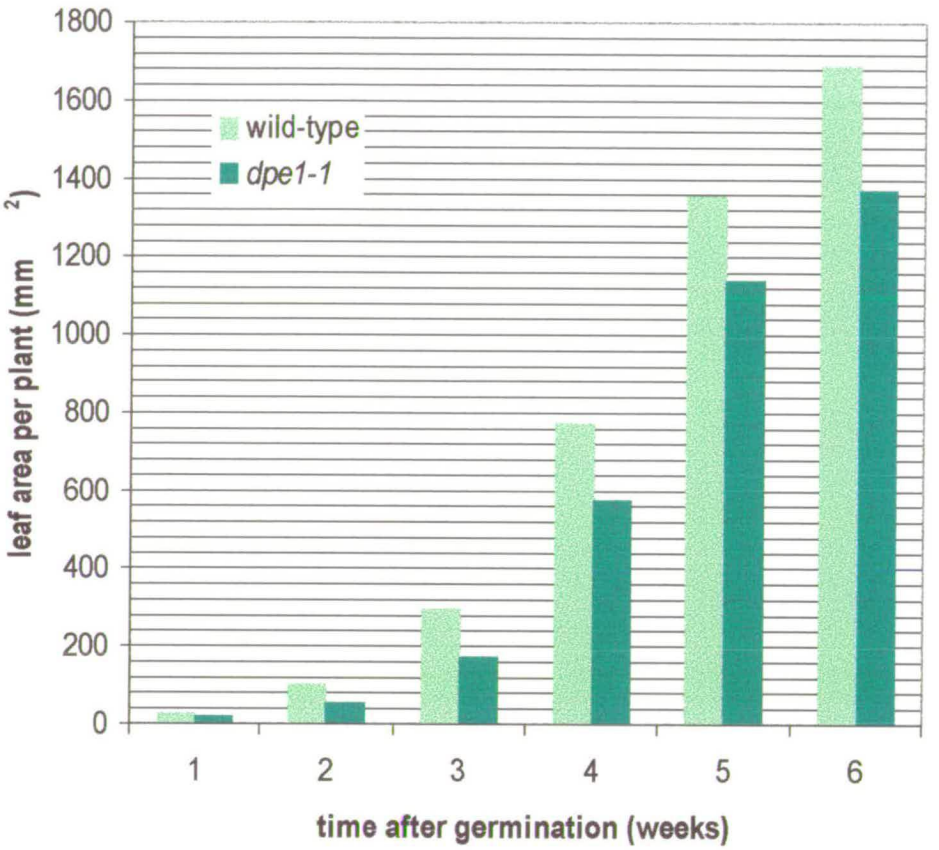



**Figure 5.8 Histogram of total leaf area produced by wild-type and mutant plants between germination and flowering**

From one week after germination, the image of ten plants was recorded using a scanner. The total leaf area was calculated from the images using ImageQuant (Molecular Dynamics).

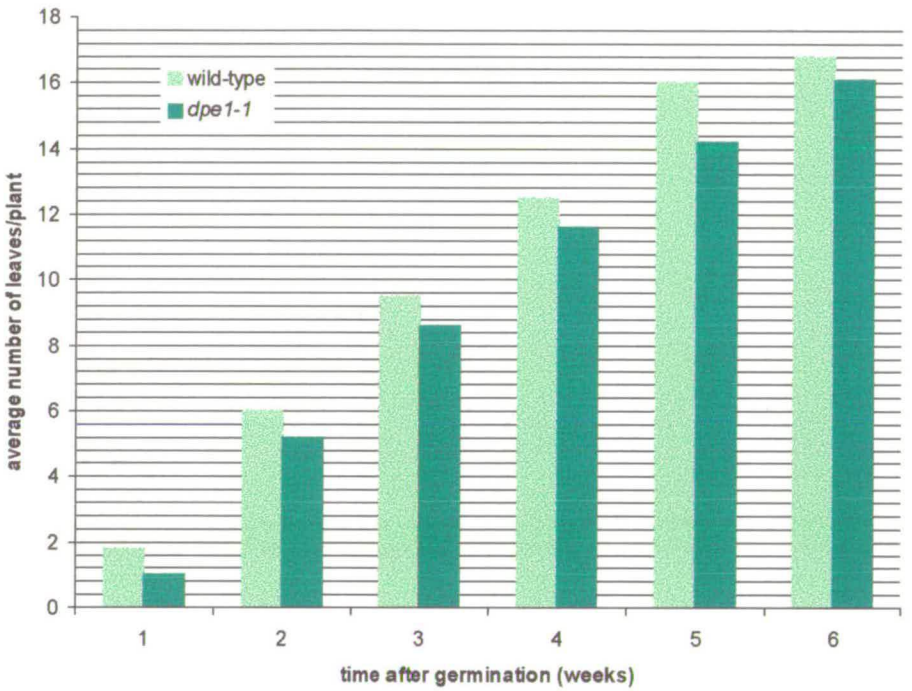



**Figure 5.9 Histogram of number of leaves produced by wild-type and mutant plants between germination and flowering**

From one week after germination, the image of ten plants was recorded using a scanner. The number of leaves (excluding cotyledons) was counted for each individual plant.

## 5.6 Analysis of starch accumulation and turnover during day/night cycle

### 5.6.1 Determination of starch content by iodine staining

The presence of starch in the leaves at the end of the day and the end of the night was determined by iodine staining. Plants were germinated and grown on soil in a growth cabinet under a 10 h photoperiod until four to five weeks old. At the end of the day (just before the lights switched off) and at the end of the night (just before the lights switched on), plants were harvested, decolourised with boiling ethanol and stained with iodine. Figure 5.10 shows the effect of the D-enzyme mutation on the turnover of starch over a diurnal cycle. *dpe1-1* plants harvested at the end of the day stain more intensely with iodine, than wild-type plants. *dpe1-1* plants harvested at the end of the night also stain considerably whereas the wild-type plants do not stain at all.

### 5.6.2 Effect of D-enzyme disruption on accumulation of starch, malto-oligosaccharides and sugars over a 24 h period

The effect of the mutation in the D-enzyme gene on the levels of starch, malto-oligosaccharides and sugars in the leaves was investigated over a 24 h period. Plants were germinated and grown on soil in a growth cabinet under a 12 h photoperiod until four to five weeks old. Over a 24 h hour period, plants were harvested, frozen and maintained on dry ice until all samples has been collected. Tissue was used for western analysis (see previously) and for biochemical analysis. Measurements of the starch, malto-oligosaccharides and sugars in these plants were made by Professor Alison Smith and Dr. Sam Zeeman at the John Innes centre, Norwich and are shown in Figures 5.11, 5.12, 5.13 and 5.14. In wild-type plants, maximal starch content was achieved at the end of the light period with breakdown occurring at the start of the dark period. In the D-enzyme mutant the starch content continued to rise for two hours after the start of the dark period. The maximum total starch accumulated was the same or marginally higher in the mutant plant than in the wild-type. However, a basal level of starch of more than 40 % of the total starch remains in the mutant at all times. There was little difference between sucrose content of the leaves of the mutant and wild-type plants, but there were elevated levels of hexoses in the leaves of the mutant particularly at the start of the day. During the day, the level of malto-oligosaccharides in the leaves of the mutant was comparable with wild-type, if slightly higher. With the onset of the dark period, a massive rise in

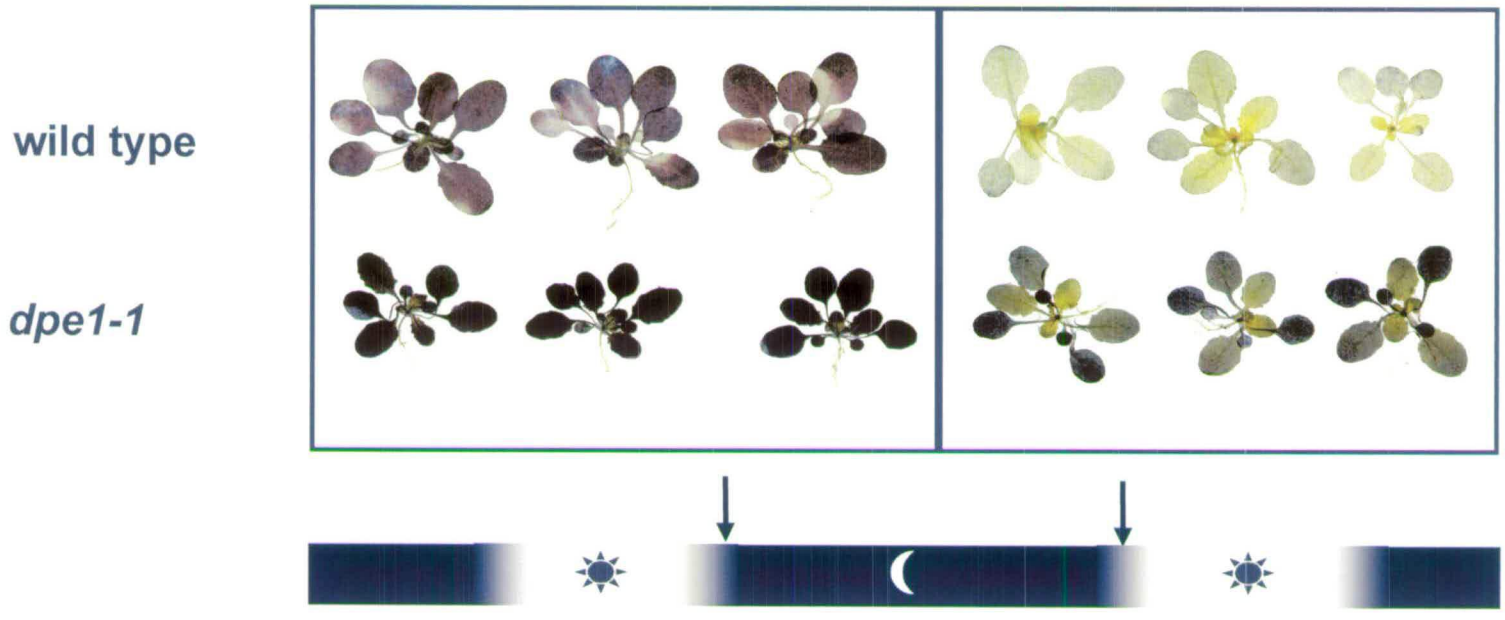



**Figure 5.10 Starch content of wild type and mutant plants at the end and beginning of a photoperiod**

Plants were harvested at the end of a 10h photoperiod and before the start of the next, decolourised in boiling ethanol and stained with iodine. Blue colour indicates the presence of starch.

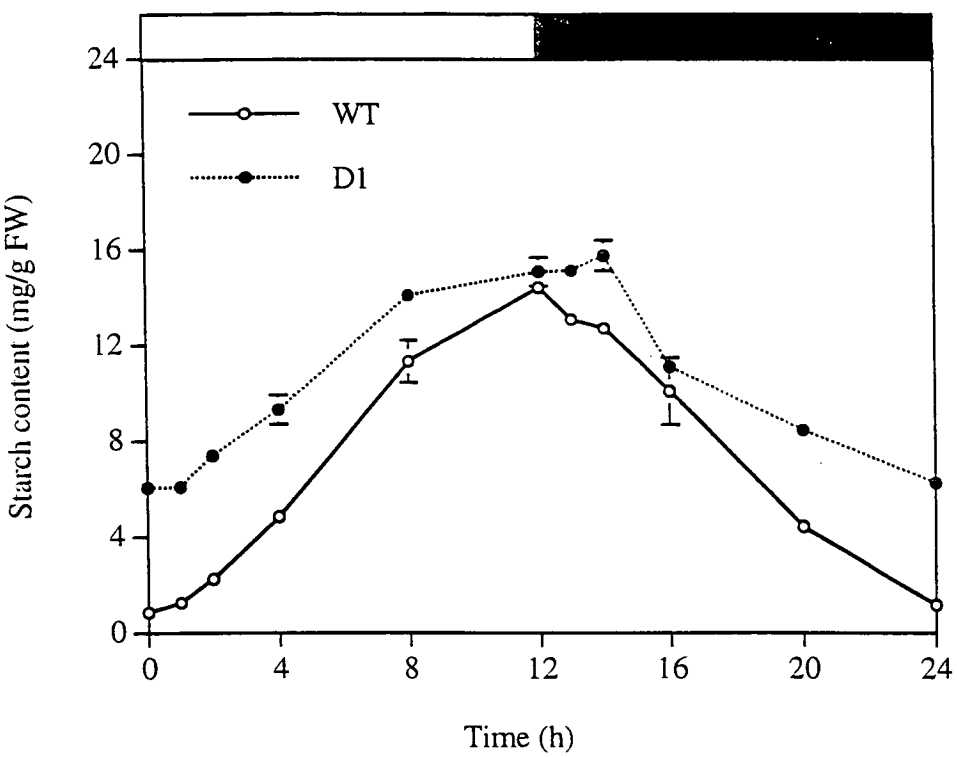



**Figure 5.11 The starch content of leaves of Ws wild-type and the D-enzyme mutant during the day and night**

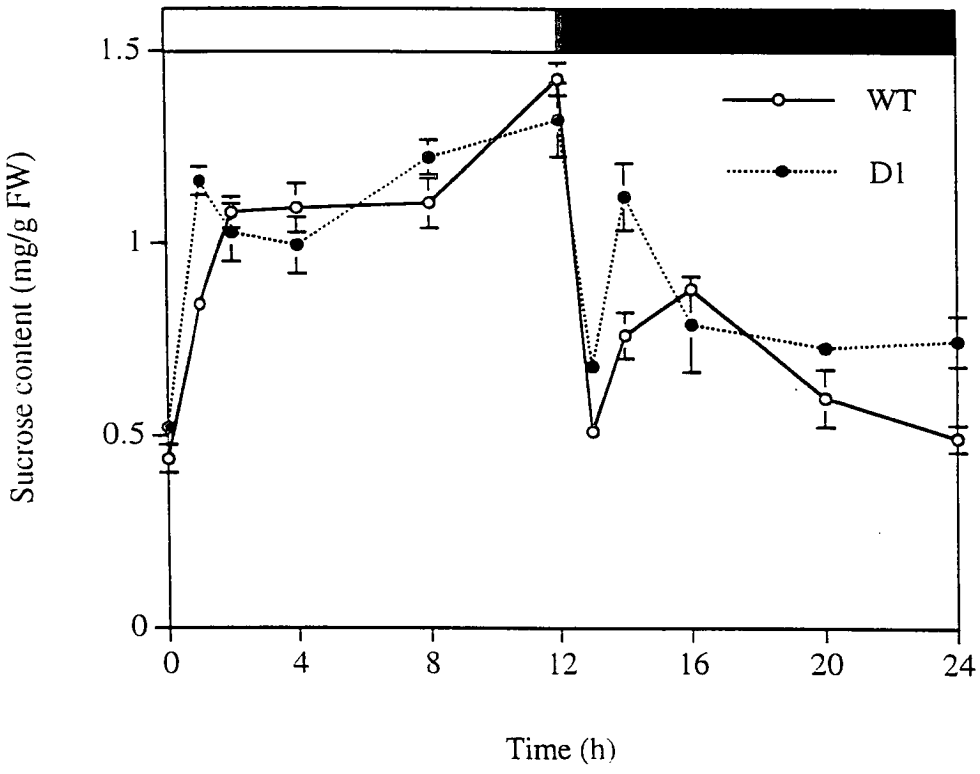



**Figure 5.12 The sucrose content of leaves of Ws wild-type and D-enzyme mutant during the day and night**

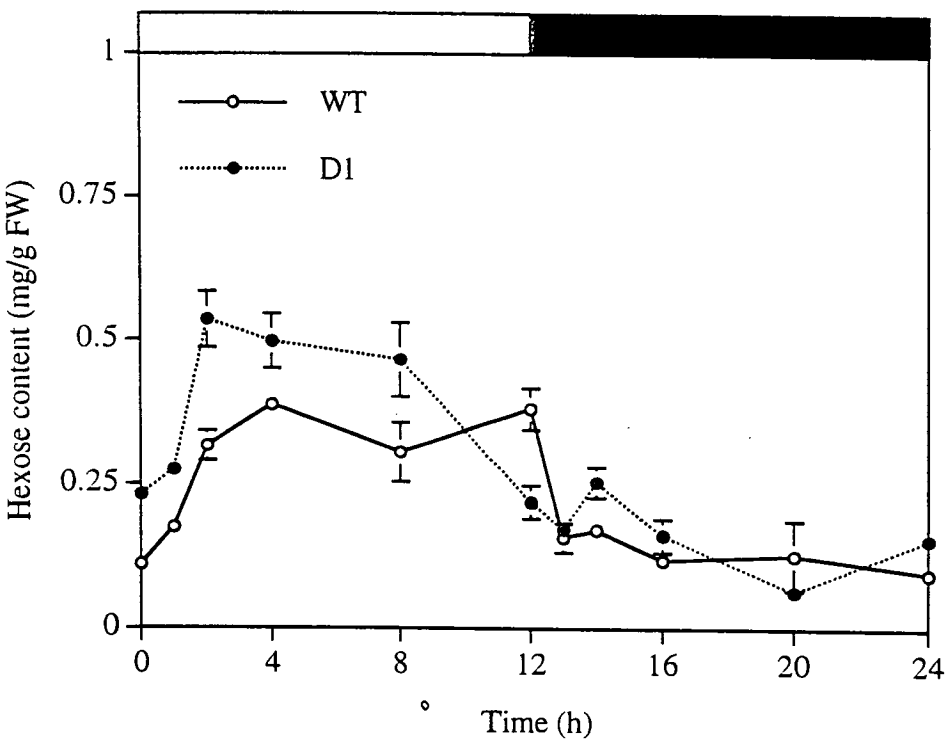



**Figure 5.13 The hexose content of leaves of Ws wild-type and D-enzyme mutant during the day and night**

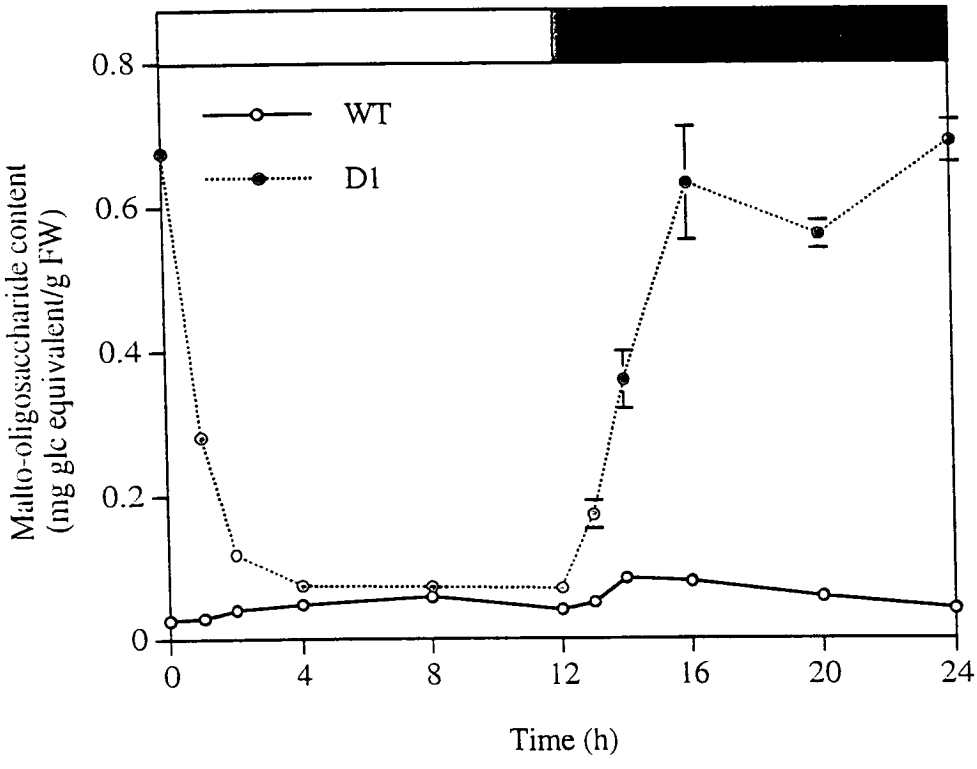



**Figure 5.14 The maltooligosacharride content of leaves of Ws wild-type and D-enzyme mutant during the day and night**

malto-oligosaccharides (MOS) occurred in the mutant plants, of more than seven times the normal physiological amounts. Although during the first two hours of the light period this elevated level of MOS in the mutant rapidly diminished, levels of MOS did not return to basal level until 4 h after the end of the dark period.

### 5.7 Analysis of starch accumulation and turnover during development by iodine staining

To investigate change in starch content during development, flowering plants with buds, flowers and siliques of different ages were iodine-stained. Figure 5.15 shows a wild-type plant seven to nine weeks old harvested mid-photoperiod and stained. This method provides a rough estimate of the starch content of organs other than leaves and in the developing embryo. The rosette leaves stain intensely and are included for comparison. In the inflorescence, significant staining occurs in the flower buds; siliques early in development stain uniformly dark (near the top of the plant), then as the embryo develops, discrete dark staining spots are visible in the siliques corresponding to the embryos (lower down the inflorescence). The inflorescence stem stains poorly as do the petals. D-enzyme mutant plants show the same pattern of staining (data not shown).

### 5.8 Effect of the D-enzyme mutation on leaf starch granule morphology

Starch was extracted from leaves of wild-type and *dpe1*-1 mutant plants harvested at the end of the day and the end of the night. Starch granules were gold-coated and analysed under the scanning electron microscope (Figure 5.16). Although granules extracted from the mutant leaves appear of a similar size to those from the wild-type, they have a more rounded appearance than the flattened discoid shape typical of wild-type granules, whether extracted at the end of the day or the end of the night.

**Figure 5.15 Starch content of organs of a flowering *Arabidopsis* plant**

Plants of ~8 weeks old were harvested mid-photo-period, decolourised with boiling ethanol and stained with iodine solution. Dark blue colour indicates the presence of starch. Figure shows wild-type plant (mutant plants appeared the same at this stage of development so is not shown).

In particular, note staining in the siliques, which progresses from homogenous dark colour in the youngest siliques (top) to discrete spots of dark staining as the embyros in the siliques develop (bottom).

**Figure 5.16 Effect of the D-enzyme mutation on starch granule morphology**

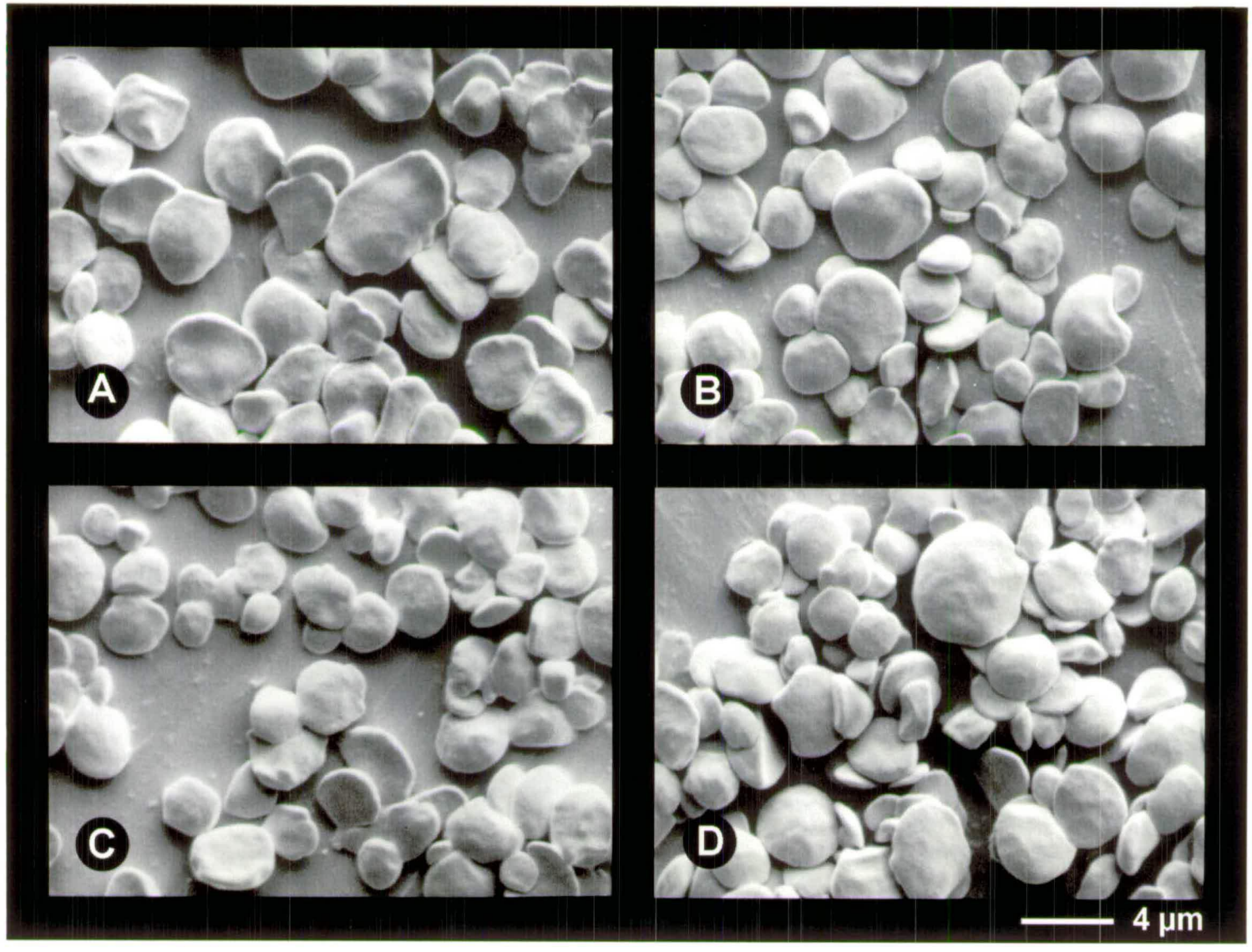
Starch granules were prepared from leaf tissue and dried onto aluminium stubs, gold sputter-coated and analysed by SEM.

A. starch granules from wild-type plants harvested at the end of the day.

B. starch granules from *dpe1-1* plants harvested at the end of the day.

C. starch granules from wild-type plants harvested at the end of the night.

D. starch granules from *dpe1-1* plants harvested at the end of the night.


A
B
C
D
4 µm

# Chapter 6

# Discussion of Results

### 6.1 D-enzyme expression in *Arabidopsis*

Flowering *Arabidopsis* plants stained with iodine indicate the presence of large amounts of starch in green leaves and sepals. As expected, starch was detectable in photosynthetic tissues where it is made in the chloroplasts. Within older green siliques, developing seeds stained as discrete dark spots. Starch accumulates in the developing seed but is presumably metabolised before the seed is fully mature, since mature seed does not stain with iodine even when crushed to a powder.

In *Arabidopsis* under normal conditions, D-enzyme expression was detectable throughout the plant with the exception of the roots. Neither starch nor D-enzyme was detectable in roots except when induced with 3% sucrose. Some large starch grains should be present in the root to fulfil a role as statoliths in the gravitropism response of the plant (starch-deficient *Arabidopsis* show reduced plastid sedimentation and reduced gravity perception consistent with the starch-statolith model – Kiss *et al.,* 1999; MacCleery and Kiss, 1999), so this method of starch detection appears insufficiently sensitive to detect these. Some D-enzyme activity is expected to be associated with the statolith starch grains but this may be too minor to detect (western blots were performed using normal sensitivity, not high sensitivity chemiluminesce reagents). D-enzyme is expressed highly in roots when sucrose is present in the growth medium. Sucrose has been shown to activate expression of a number of starch synthetic genes.

D-enzyme was expressed where starch accumulates in *Arabidopsis*. D-enzyme expression declined in senescing leaves and also senescing siliques (consistent with a role in starch turnover during seed development, discussed later). In addition, it also seemed to be expressed in some other regions where starch is less abundant. Expression of D-enzyme protein was high in all green tissues including the inflorescence stem. Stem does not stain well with iodine indicating that it contains comparatively little starch.

In studies of inflorescence gravitropism in *Arabidopsis*, sedimentation of plastids was observed in inflorescence stem endodermal cells but not in a starchless mutant

(Weise *et al.*,1999). D-enzyme was also found to be expressed in flower petals yet these too contain little starch as detectable by this method.

All D-enzyme activity detectable in *Arabidopsis* wild-type plants by the methods used in this study was entirely attributable to the product of the *DPE-1* gene. This gene was cloned and found to have a high degree of homology with the potato D-enzyme sequence (Takaha *et al.*, 1993). The coding sequence and the promoter region both contain sequences which are evolutionarily conserved with the α-amylase family of enzymes.

The cDNA encoding D-enzyme contains sequence at the 5' end which has the characteristics of a plastid targeting peptide. The mature protein detected in crude extracts of *Arabidopsis* had a molecular weight about 5000 Daltons smaller than that calculated from its amino acid content and this is consistent with cleavage of a transit peptide. At least 80% of D-enzyme activity in *Arabidopsis* leaves is reported as chloroplastic (Lin *et al.*, 1988). The promoter region also contains sequences conserved in plastid-related genes. Immuno-gold labelling of wild-type leaf tissue sections showed that D-enzyme 1 was located within the chloroplast since negligible label was found outside. Due to the size of the label, depicting chloroplast and cytosol in one photograph proved difficult; this could be facilitated by using silver-enhancement of the gold-label. Label was found associated with the thylakoids and starch grains (however since some label was also found on the starch grains of the mutant plant some starch grain labelling must be non-specific). This evidence supports the association of D-enzyme with the chloroplast. That being the case, it raises questions as to whether D-enzyme is associated with plastids in the petals (and why it should be present in petals at all).

There are a number of sequences in the promoter region of the *DPE-1* gene that indicate that gene expression may be regulated by light. However, over a 24 h period levels of expression of D-enzyme protein remained constant. This was also reported for D-enzyme in potato (Takaha, 1996) and there are a number of other enzymes of starch metabolism for which this is the case. It is as yet unknown whether its activity also remains constant over this period. The enzyme may be subject to post-

translational regulation or be regulated by the availability of substrates. It is also possible that it is in constant requirement if it is involved in both starch synthesis and degradation.

A second D-enzyme-like sequence in *Arabidopsis* was made available by the genome sequencing project. This sequence has lower homology to D-enzyme 1 than potato D-enzyme, yet contains three of the four conserved regions found in glucanotransferases of the α-amylase family. It also contains a potential (αβ) 8-barrel core structure and a conserved hand-shaped loop region, from which it is predicted to be a glucanotransferase not a hydrolase, and unable to act on cyclodextrins (Ingo Przylas, unpublished). The coding sequence does not include an obvious transit peptide indicating that the peptide would not be directed to the chloroplast. Many enzymes of starch metabolism (including α- and β-amylase, starch phosphorylase and debranching enzyme) occur as plastidial and extraplastidial forms. D-enzyme activity has been found in both chloroplastic and cytosolic fractions in *Pisum sativum* and *Spinacia oleracea*, and in *Arabidopsis* D-enzyme activity was eluted as two major peaks. It is possible that this sequence encodes an extraplastidial form of D-enzyme in *Arabidopsis*. If the protein is expressed it does not appear to be immunogenically related to D-enzyme 1. No residual D-enzyme activity is detected in the *dpe1-1* mutant plants, implying that this second putative D-enzyme may be expressed at undetectably low levels, or not expressed under the circumstances of this study, or that conditions required for its activity are quite different from that of other D-enzymes. The sequence shows more homology to the bacterial amylomaltase sequences than to those of D-enzymes of higher plants. The sequence could thus be of ancient prokaryotic origins and may now be redundant.

### 6.2 An *Arabidopsis* plant completely lacking D-enzyme

Previously no mutant for D-enzyme had been isolated from any higher plant. The effect of reduced D-enzyme activity on plant growth and development was investigated in transgenic potato plants with only 1% of wild-type D-enzyme activity (Takaha *et al.*, 1998). These plants grew more slowly, produced fewer leaves than the wild-type and also produced necrotic apical shoots. The plants also showed a late

flowering phenotype and flower buds sometimes abscised before opening (Takaha, 1996). Despite the phenotypic changes associated with decreased D-enzyme activity, starch appeared normal suggesting that 1% of D-enzyme activity may be sufficient for apparently normal starch metabolism.

An *Arabidopsis* plant null mutant for D-enzyme was isolated and shown to contain little detectable D-enzyme activity (<6.7% of wild-type) by biochemical assay and no detectable protein by western blotting. Since another sequence had been isolated which encoded a putative D-enzyme in the *Arabidopsis* genome sequencing project it was important to determine that this enzyme was not functioning *in vivo* to compensate for the absence of D-enzyme 1. No reduction was found in the activities of other starch-degrading enzymes in crude extracts of leaves of *dpe1-1* compared with those of wild-type (Table 5.1). There was a small but significant increase in the activities of β-amylase and starch phosphorylase in the leaves of *dpe1-1* relative to wild-type (Student's *t* test; $P<0.05$). These pleiotropic effects have been reported as characteristic of *Arabidopsis* mutants with lesions in starch metabolism (Caspar *et al.*,1989; Zeeman *et al.*, 1998).

The *dpe1-1* mutant was isolated from a collection of plants containing T-DNA insertions. Crossing the mutant plant with wild-type and determining the number of kanamycin sensitive progeny suggested that the mutant plant might contain an additional T-DNA insert not associated with that disrupting the D-enzyme gene, although this has yet to be confirmed. In Southern blots, hybridising restriction fragment patterns in a number of independent mutant plants were identical to each other regardless of the enzyme used. This strongly suggests that the *dpe1-1* mutant contains only the T-DNA insertion in the D-enzyme gene. However, the wild-type control used for all comparisons in this study has been obtained from the heterozygous parent of the D-enzyme null mutant and so has the same genetic background as the mutant. If an additional T-DNA insertion was present in the *dpe1* line used for this study the probability that its presence might result in the same starch-excess phenotype expected for that of a D-enzyme mutant would be extremely low.

### 6.3 The role of D-enzyme in starch metabolism

Seeds from wild-type and mutant plants germinated at the same time, but after one week of growth the wild-type plants had produced their first leaves and the mutant plants had not. D-enzyme is clearly not necessary for viability, but may be necessary for proper development of the seed. Wild-type seed, as stated previously, contains no detectable starch. Simple experiments where seeds were ground with iodine solution and the iodine-starch complex sedimented by centrifugation revealed that the *dpe1-1* seed does contain starch, suggesting that D-enzyme may be necessary for metabolism of starch during seed development. In the developing embryos of *Brassica napus*, starch accumulated in the silique during the early-mid stage of cotyledon development (i.e. during the early part of oil accumulation) and then declined, with the mature embryo containing almost no starch (da Silva *et al.*, 1997). The D-enzyme mutant seed might contain less lipid reserve and instead contain starch that it is unable to utilise for early growth. In the light of a possible co-ordinate role for D-enzyme with plastidic starch phosphorylase it is interesting to note that *in-situ* hybridisation revealed a correlation between phosphorylase gene expression and starch granule formation in cotyledon development (Buchner *et al.*, 1996).

The *dpe1-1* mutant plants grew more slowly than wild-type: photosynthetic area and fresh weight of plants was lower and the plants also produced fewer leaves. The mutant plant lagged behind the wild-type plant by approximately one pair of leaves throughout its development, from as early as one week after germination. This may be partly due to the poor development of the embryo in the mutant plant which is consequently developmentally delayed by one pair of leaves. The difference in total leaf area between the mutant and the wild-type plants is more pronounced than can be accounted for by the development of one pair of leaves and indicates that the lack of D-enzyme decreases the daily growth of the plants. During the first five weeks of growth weight of the mutant plant lagged behind the wild-type by 25-50%. As the plants approached flowering the difference between them decreased and the individual leaves of the mutant reached normal size, although they appeared thinner than those of wild-type plants. At seven weeks when the plants were flowering and

the rosette leaves were beginning to senesce, the weights of mutant and wild-type plants were similar. The *dpe1-1* mutant plants were slightly delayed in the onset of senescence, which may explain why they appear to 'catch-up' with the wild-type. Fresh rather than dry weight was used as a measure of growth despite the fact that this measurement includes not only fixed carbon but a large percentage of water which could vary between the mutant and wild-type. However, no difference in turgor was noted between the two. Plants flowered at 6 weeks old even when germinated and grown in a 10 h photoperiod and this may be the result of low light quality. This may be masking a late flowering phenotype of the D-enzyme mutant which was seen with the transgenic potato plants, and other *Arabidopsis* starch metabolic mutants (e.g. *pgm, sex1, cam1).* The late flowering phenotype in starch mutants is thought to result from an inability of the plant to mobilise starch in order to produce a sucrose signal when photosynthesis is limited at floral induction (Corbusier *et al.,* 1998).

*Arabidopsis* plants lacking D-enzyme have a 'high starch' phenotype. In wild-type leaves starch is accumulated during the light period and then metabolised completely during the following dark period. This can be seen in plants stained for starch at the end of the day and at the end of the night (Figure 5.10). Amylose reacts with iodine to give a bluish colour while the colour given with amylopectin is a reddish-brown. These colours relate to the relative affinity that the polymers have for iodine. However, long chain amylopectins (with chain lengths greater than 20 DP) also have a high affinity for iodine (Takeda *et al.*, 1987). The dark blue staining can only be interpreted therefore as high starch rather than high amylose on the basis of this experiment. The light areas are the result of some leaves overshadowing others. The mutant plants stain more intensely for starch at the end of the day but more noticeably they also contain starch at the end of the night. The mutant plants are clearly smaller than wild-type so to ensure that the intense staining at the end of the day was not an artefact caused by size, some unusually small wild-type plants were also stained to confirm this result (data not shown). Starch granules were prepared from mutant and wild-type leaves harvested at the end of the day and the end of the night and analysed by scanning electron microscopy. Although the granules were

similar in size, the wild-type *Arabidopsis* starch granules had the typical flat ovoid appearance, whereas the mutant starch granules had a fatter, more rounded shape. This result is consistent with an excess starch phenotype.

There are two possible explanations for the starch accumulation in the mutant. Firstly, that D-enzyme may be necessary for the breakdown of starch during the night. It has been previously suggested that D-enzyme, together with plastidic starch phosphorylase, is involved in the complete breakdown of malto-oligosaccharides during starch breakdown in the plastid. Glucans are broken down by starch phosphorylase no further than malto-tetraose and it is proposed that D-enzyme converts malto-oligosaccharides into longer molecules which can be further attacked by starch phosphorylase. Secondly, from the structure of starch granule it is clear that different types of starch structure may be more or less accessible to degradation - 'rapid turnover' starch and 'slow turnover starch'. It is thought that the material in transitory starch which is not turned over on a regular basis may resemble storage starch in that it contains amylopectin of high molecular mass and large amounts of amylose. An alternative explanation for the high starch phenotype is that D-enzyme might be necessary for the synthesis of the high turnover starch. In the absence of D-enzyme, starch synthesis might be directed towards the synthesis of the slow turnover type of starch which consequently accumulates since it is metabolised less rapidly.

In the developing embryos of *Arabidopsis,* D-enzyme could be involved in the synthesis/breakdown of transient, rapid turnover starch necessary for the synthesis of lipids and sugars required later in seed development. An absence of the enzyme thus might result in slower and consequently incomplete degradation of the starch leading to a reduction in available lipids and ultimately, to a poorer embryo. Plastids isolated from oilseed *Brassica napus* embryos have the capacity for both starch synthesis and degradation throughout the phases of net starch synthesis and net starch degradation. da Silva *et al.* (1997) suggest that starch may be turned over throughout embryo development and that changes in content reflect the net balance between synthetic

and degradative capacity rather than a synthetic phase followed by a degradative phase.

Recently, a D-enzyme mutant was isolated from *Chlamydomonas reinhardtii*, a starch-storing unicellular algae. *C.reinhardtii* accumulates a polysaccharide similar to cereal endosperm storage starch when grown under nitrogen starvation conditions. Transitory starch synthesis is obtained under conditions of active photosynthesis and cell division. Colleoni *et al.* (1999b) define the *sta11-1* mutation as partly conditional – it is expressed in both conditions but transitory starch synthesis is affected to a lesser degree. Maximal expression of the defect is obtained in starvation conditions where 'decreases in starch amounts exceeding 90% together with increases in amylose percentage and malto-oligosaccharide content are obtained'. These data are difficult to compare with the *dpe1-1* mutant since *Arabidopsis* does not accumulate storage starch and the *sta11-1* mutant does not show the reduced starch phenotype strongly except under conditions of starvation. However, the high amounts of unbranched malto-oligosaccharides and increases in amylose content were observed when the *sta11-1* mutant is grown in undepleted medium.

The starch content of leaves of wild-type and *dpe1-1* mutant *Arabidopsis* was measured during the day and the night (Figure 5.11). In wild-type plants, maximal starch content was achieved at the end of the light period with breakdown occurring at the start of the dark period. In the D-enzyme mutant the starch content continued to rise for two hours after the start of the dark period. The maximum total starch accumulated was the same or marginally higher in the mutant plant than in the wild-type. However, a basal level of starch of more than 40 % of the total starch remains in the mutant at all times. This starch is seen in the plants stained with iodine at the end of the night. From these data, the rate of both starch synthesis and starch breakdown are reduced in the mutant plant, as is the total amount of synthesis. There was little difference between sucrose content of the leaves of the mutant and wild-type plants, but there were elevated levels of hexoses in the leaves of the mutant particularly at the start of the day (Figures 5.12 and 5.13).

The malto-oligosaccharide content of the leaves was also determined over a 24 h period (Figure 5.15). During the day, the level of malto-oligosaccharides in the leaves of the mutant was comparable with wild-type. With the onset of the dark period, a massive rise in malto-oligosaccharides (MOS) occurred in the mutant plants, of more than seven times the normal physiological amounts. This rise in MOS must be a direct consequence of the absence of D-enzyme. Since net degradation begins at the onset of the dark period, and the excess MOS are not produced during the day in the mutant, this strongly suggests these MOS must be produced as a consequence of degradative processes, and that D-enzyme is involved in degrading the MOS as the next step in the process of starch breakdown. The decrease in the amount and rate of starch synthesis could be an effect of the excess MOS. It is unknown as yet whether phytoglycogen accumulates in the mutant as a result of synthetic activity acting on MOS, since elevated levels of MOS are still present at the start of the light period. The reduction in the high levels of MOS at the start the day (within the first 2 h), in the absence of D-enzyme, is probably due to degradative activity (by starch phosphorylase, or α-amylase) and may result in elevated levels of hexoses within this time period (degradation of phytoglycogen might also cause this result). The basal level of starch is probably amylose which could be a result of the stimulation of amylose synthesis by the high levels of MOS (Denyer *et al.*, 1997).
This does not completely rule out a role for D-enzyme in synthesis of starch, but since very little excess MOS accumulate during the day in the absence of D-enzyme, in *Arabidopsis*, it is highly unlikely that MOS are a substrate for D-enzyme for incorporation into amylopectin as part of a major pathway of biosynthesis as proposed by Colleoni *et al.* (1999b).

### 6.4 Future work

Further biochemical analysis of the *dpe1-1* mutant is necessary. In particular, analysis of the starch, both that accumulating at a basal level and that turned over during a diurnal cycle. The proportion of amylose, the molecular weight of debranched amylopectin and the branch chain lengths of amylopectin should be determined. It would be of interest to determine if the mutant plant accumulates phytoglycogen.

It is unclear why the rate of starch synthesis is slower in the mutant or what causes the delay in the switch from net synthesis to net degradation at the light/dark transition. Since the process involves co-ordinate activity of a number of enzymes (the roles of which are also uncertain) further investigation into these areas may need to be studied in double, or triple mutants, for example with starch phosphorylase or debranching enzyme mutations. A double mutant will be made with the *dbe1* (SZ20) debranching enzyme mutant in the lab of Professor Alison Smith, which may contribute to information on whether D-enzyme has a role in starch synthesis as well as in breakdown. It is important in the crossing of these mutants that there are no other insertions in the D-enzyme mutant. This should be clarified prior to crossing.

New resources have recently become available facilitating the isolation of more mutant plants. It is now possible to obtain membranes containing an array of pools of DNA from thousands of T-DNA tagged mutant plants which can be screened merely by hybridisation with an EST sequence, without the need for specific primers and many PCR reactions. In addition, many more lines of mutants have been generated increasing the chances of obtaining a mutant in the particular gene of interest.

It is probable that *DPE-1* constitutes the major D-enzyme activity in *Arabidopsis*. However, the possibility that there are other, perhaps minor, activities cannot be excluded. Since a second D-enzyme sequence was revealed by the genome sequencing project, it would be of interest whether this isoform has activity and/or importance in starch metabolism. This could be approached by isolating a mutant for the *DPE-2* gene. Using the sequence provided by the database it should be possible to design primers for amplification of the sequence by PCR from genomic DNA to use as a probe. Alternatively, an EST may be available. cDNA sequence could be obtained using the primers to amplify from the *Arabidopsis* cDNA library prepared in Chapter 3. If a full length sequence was obtained it may be possible to obtain functional protein, in which case its activity *in vitro* might be investigated. The full *Arabidopsis* genome sequence may be soon be made available, at which point it will reveal if there are other glucanotransferase activities.

As previously discussed, it has been proposed that there may be co-ordinate activity between D-enzyme and plastidic starch phosphorylase. In Chapter 3 the construction of an *Arabidopsis* cDNA library was described. The library was enriched for sequences for which transcription is induced by high levels of sucrose. Using a potato starch phosphorylase probe, two cDNAs were isolated from this library and were shown to have a high degree of homology to the plastidic and cytosolic isoforms (L and H) of starch phosphorylase (this work is not described in this study). These sequences have yet to be identified as part of the genome sequencing project. These sequences may enable the isolation of mutants for starch phosphorylase.

Both genomic and cDNA clones have been isolated for the *DPE-1* sequence. Constructs could be made placing the gene under the control of an inducible promoter. The *dpe-1* mutant plants could be transformed with such a construct, enabling firstly complementation to be demonstrated and secondly to provide a useful tool for investigating starch turnover in plants which are normal in growth and starch content prior to the experiment. Mutant plants have a high basal level of starch which is probably accumulated over time and, like that accumulating in older leaves in wild-type plants, may resemble that of storage starch. If this kind of inducible mutant was used to create double mutants it may greatly facilitate interpretation of the phenotypes of crosses.

Molecular dissection of enzymatic pathways is clearly a powerful approach to determining the *in vivo* role of component enzymes. Production of mutants coupled with rigorous biochemical analysis may provide some of the long unanswered questions regarding the synthesis and degradation of the starch granule.

Information about the effect of mutations on the structure of starch may also be of interest to the fields of agriculture and industry. Mutations under the control of inducible organ specific promoters may enable increases in the amount of starch in crops plants. Mutations which alter the structure of starch could be useful if the properties of the starch were better suited to industrial requirements. Starch, which required less physical processing and chemical modification would reduce energy

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# Bibliography

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